

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

EXPRESS MAIL CERTIFICATE

Date 5/29/01 Label No. 56706741126Us

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

PLEASE CHARGE ANY DEFICIENCY UP TO \$300.00
OR CREDIT ANY EXCESS IN THE FEES DUE WITH
THIS DOCUMENT TO OUR DEPOSIT ACCOUNT NO. 04-0100

Name (Print) D B Peck

Signature D B Peck

Customer No.: 

07278

PATENT TRADEMARK OFFICE

1225/0C674

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David BERD

Serial No.: 08/203,004

Art Unit: 1642

Filed: February 28, 1994

Examiner: Susan UNGAR

For: COMPOSITION AND METHOD OF USING TUMOR CELLS

DECLARATION OF DONALD P. BRAUN, PH.D.
UNDER 37 C.F.R. § 1.132

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, Donald P. BRAUN, hereby declare and state as follows:

1. I am a citizen of the United States of America and am more than 21 years of age.

2. I presently hold the title of Administrative Director of the Medical College of Ohio Cancer Institute and Professor of Surgery at the Medical College of Ohio, 3120 Glendale Avenue, Toledo, Ohio, where I have been employed since 1999. Prior to this position, I held the positions of Director, Scientific Program Development and Professor of Medicine and Immunology/Microbiology at the Rush Cancer Institute, Rush Medical College, Chicago, Illinois. I hold a Ph.D. and M.S. degrees from the University of Illinois at the Medical Center, Chicago, and a B.S. from the University of Illinois, Urbana. I have over 25 years research experience in immunology, microbiology, and oncology, particularly cancer immunology. My qualifications are set forth more fully on the copy of my *Curriculum Vitae*, attached as Exhibit A.

3. My only connection with Avax Therapeutics, Inc. ("Avax"), is as a clinical researcher. I understand Avax has licensed certain patents and patent applications by Dr. David Berd (solely or with others) related to haptenization of tumor cells to generate an effective anti-tumor immunotherapy ("technology") from Thomas Jefferson University. I am not an employee or shareholder of Avax.

4. I know Dr. David Berd professionally. However, we have not collaborated on any research.

5. The law firm of Darby & Darby, attorneys for Applicant, has retained my services as an expert in connection with prosecution of these patent applications. In connection with these services, I attended and participated in a personal interview with the Examiner on

January 5, 2001. The law firm is compensating me for my services. Thus, I have no personal interest in Avax or the patent applications.

6. I have read and am familiar with Berd et al., Proc AACR 1989;20:382 (hereinafter "Berd 1989"; a copy is attached as Exhibit B). In particular, it is my understanding that the claims of the above-identified application have been rejected in part because the Examiner believes that Berd 1989 teaches a successful method of inducing an antitumor response comprising regression of a metastatic melanoma tumor by administering cyclophosphamide prior to autologous, irradiated, DNP-conjugated melanoma cells in combination with BCG.

7. In my view as one of skill in the art in this field, the Berd 1989 abstract does not describe successfully treating melanoma tumors with a haptenized melanoma tumor cell immunotherapy vaccine. The abstract, like most of the abstracts presented at the AACR meetings, optimistically reports preliminary observations from a new protocol. Because the abstract omits certain details, and because by its own terms the results are preliminary, one of ordinary skill in the art would not be able to conclude from this Abstract that one could effectively treat melanoma, much less any other type of cancer. Nothing in the Berd 1989 abstract suggests that this approach addresses fundamental questions of tumor vaccination (*e.g.*, as posed in a 1993 review on tumor vaccination written by myself and Jules Harris, M.D. for the Biotechnology Journal (Volume 1, No. 3), entitled "Cancer-Concept to Clinic" (Exhibit C)); which type of immune response are most important in a host response to cancer (Exhibit C, p. 28 and Table 1); whether whole cells or extracts should be used (*Id.*, pp. 28-29); whether to use adjuvants or cytokines (*Id.*, p. 29); and whether an

antitumor response would lead to autoimmunity (Id.). Furthermore, with respect to whole cell vaccines, whether to use autologous or syngeneic cells; fresh surgical specimens or cell lines; irradiation; reproducibility; and other factors (Id. P. 29, Table 2). The haptenization protocol of the Berd 1989 Abstract not only fails to address these variables, but also raises a new issue. Consequently, in 1989, one of skill in the art would not have viewed Berd 1989 as establishing an effective protocol for cancer immunotherapy.

8. By way of background, as described during the interview, early work on developing tumor vaccines in animal models yielded successes far beyond the reality for humans. Animals used in these models are typically immunocompetent, and the tumor cell lines (unlike spontaneous tumors) bear one or more strongly immunogenic antigens. Under these circumstances, the ability to generate an immune response cannot be viewed as particularly surprising. Unlike animal models, human cancer patients are typically immunosuppressed, whether from the tumor or chemotherapy. Spontaneous human tumors are weak immunogens. Thus the trick is to determine how to break tolerance and elicit immunity in a human subject. In 1992, Hanna and colleagues proposed one route, albeit based on animal data; but their results were inconclusive (Exhibit C, p. 30). Berd and colleagues offered another approach, pretreatment with cyclophosphamide to inhibit suppressor T lymphocytes (Id.) In the context of these multiple approaches, it was, in 1989, unknown and unknowable whether haptenization was a viable approach to elicit immunity to unhaptenized melanoma cells, much less that the approach could have therapeutic potential.

9. In my view as one of skill in the art in this field, the Berd 1989 abstract does not provide a definitive protocol. The description of the vaccine is ambiguous, stating that 10-25 million cells are used. It does not state if these are given as a single injection or divided into multiple sites, nor does it specify the route of administration (*e.g.*, intradermal, subcutaneous, or intramuscular). It does not specify if the injections are given in proximity to tumor sites or even directly into the tumor site (a location that one familiar with the literature at the time would assume from a reading of this abstract). It does not state how conjugation to DNP was performed or the extent of tumor cell substitution. It does not specify the ratio of tumor cells to BCG microorganisms. It also does not describe the schedule of vaccination beyond stating that vaccine or DNCB sensitization occurred 3 days following low dose cyclophosphamide i.v. administration. The statement "after 2 vaccine treatments (8 weeks)" is totally ambiguous. It is not clear if this represented a point 4 weeks following vaccine #2, 3 weeks following vaccine #2, 2 weeks following vaccine #2, or 1 week following vaccine #2. A vaccination schedule of every 55 days could apply to what is described as readily as any of the other schedules listed above. Hanna and Peters (Cancer Research 1978;38:204-9, attached hereto as Exhibit D) emphasize the critical importance of dose, schedule, route of administration, and ratio of viable tumor cells to BCG organisms in the outcome of autologous tumor vaccines. The Berd 1989 abstract, however, provides none of these details, nor could they be deduced. Without these details, one of ordinary skill would be unable to practice the technology predictably, and furthermore would have little incentive to view this approach as any more promising than a myriad of others.

10. ***There is no indication in the protocol that patients have developed an immune response to unmodified cells.*** The opening statement of Berd 1989 indicates that a previous method practiced by Berd using non-haptenized tumor cells induced DTH to melanoma cells. But in the Berd 1989 abstract, DTH testing was done only with DNP-modified tumor cells or DNP-modified autologous lymphocytes following patient sensitization with topical application of DNCB. The positive reactions described in the Berd 1989 abstract are not surprising given the experience of Fujiwara (J Immunol 1980;124:863-869; attached hereto as Exhibit E), Sherman (J Immunol 1979;123:501-502; attached hereto as Exhibit F), and others using haptens to sensitize hosts against haptenized target cells. However, the vaccine protocol of the invention involving intradermal injection of hapten-modified autologous tumor cells, results in DTH to autologous non-haptenized tumor cells, an event that could not have been anticipated nor expected as a result of what is described in the Berd 1989 abstract or from what was known in the literature.

11. ***There is no convincing indication that the patients described in the Berd 1989 abstract received any clinical benefit.*** The descriptions of inflammatory reactions, CD4 and CD8 infiltration, and fluid accumulation over tumor lesions is no indication of clinically significant tumor regression (defined by those practiced in the art as a greater than 50% reduction in tumor size without concomitant progression in other sites). In fact, the description of lesion changes in the patients would be expected at the time of its publication, since one would presume based on Fujiwara (Exhibit D), that the patients had been sensitized to DNCB and then injected intratumorally with DNP-modified tumor cells. A skilled immuno-oncologist would have presumed that the tumor cell vaccine, which produced the described physical changes in proximity to tumor sites after only

two vaccine treatments, had been administered by intratumoral injection as taught by others (while not practiced by the Berd protocol developed subsequent to the 1989 Abstract). The same outcome would have been seen if the patients had been sensitized to BCG and then injected intratumorally with BCG. Thus, the description of the lesion changes in the 1989 Abstract would be impossible to interpret as indicative of a clinical response to a systemic vaccine. A reader would assume that a clinically meaningful tumor regression, if present, would have been reported in the abstract, and that the absence of such a report represented uncertainty.

12. For all of these reasons, the Berd 1989 abstract does not disclose a method for the successful vaccination of cancer patients using haptenized autologous tumor cells.

13. A final basis for the above statement can be deduced from Exhibit C. As discussed above, this review cites the work by Hanna and Hoover (references 7-9), and the work by Berd et al. employing non-haptenized melanoma cells (reference 10), among a number of hopeful, even promising, research approaches to cancer immunotherapy. Had the Berd 1989 abstract been indicative of a clinically meaningful vaccine methodology, that approach would have been considered in the review as well.

14. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

code and that such willful false statements may jeopardize the validity of the instant application or of any patent issued thereupon.

Respectfully submitted,

Date: _____

5/24/01



Donald P. Braun, Ph.D.

Enclosure: Exhibit A: Curriculum Vitae of Donald P. Braun, Ph.D.
 Exhibit B: Berd et al., Proc AACR1989;20:382
 Exhibit C: Braun and Harris, Biotechnol J 1993;1, No. 3.
 Exhibit D: Hanna et al., Cancer Research 1978;38:204-209
 Exhibit E: Fujiwara et al., J Immunol 1980;124:863-869
 Exhibit F: Sherman, J Immunol 1979;123:501-502

Exhibit A

CURRICULUM VITAE

Donald P. Braun, Ph.D.

January, 2000

BUSINESS ADDRESS: Medical College of Ohio Cancer Institute
Medical College of Ohio
3120 Glendale Avenue
Toledo, Ohio, 43614
Phone: 419-383-6632
Fax: 419-383-6714
e-mail: dbraun@mco.edu

HOME ADDRESS: 14954 Stonehaven Dr.
Perrysburg, Ohio, 43551

PERSONAL: Born: New York, NY; March 7, 1950
SS # 355-44-2224
Married: Judy Braun
Children: Jennifer, Matthew, Bethany

EDUCATION AND TRAINING:

1972 University of Illinois, Urbana, IL, B.S. degree.
1974 University of Illinois at the Medical Center, Chicago, IL, M.S. degree.
1976 University of Illinois at the Medical Center, Chicago, IL, Ph.D. degree.

CHRONOLOGY OF EMPLOYMENT:

1976-1977 Research Associate, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL.
1977-1978 Instructor, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL
1978-1979 Research Associate, Section of Medical Oncology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL.
1979-1980 Instructor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1981-1983 Assistant Professor, Department of Medicine; Assistant Professor, Department of Immunology/ Microbiology, Rush Medical College, Chicago, IL.
1983-1987 Associate Professor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1987 Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.

- 1989 Associate Director, Section of Medical Oncology (for Research); Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
- 1993-1999 Director, Scientific Program Development. Rush Cancer Institute.
- 1993-1999 Professor of Medicine and Immunology/Microbiology.
- 1999-present Administrative Director of the Cancer Institute, Medical College of Ohio
- 1999-present Professor, Department of Surgery, Medical College of Ohio.

FEDERAL GOVERNMENT/PUBLIC ADVISORY COMMITTEES:

- 1982-1984 Member, Experimental Therapeutics Study Section, National Cancer Institute.
- 1983, 1985 Member, Small Business Innovation Grant Review Study Section, NCI.
- 1985-1988 Member, Experimental Therapeutics I Study Section, NCI.
- 1985 Chairman, Experimental Therapeutics Special Study Section, NCI.
- 1985-1992 Biological Response Modifier Committee, Illinois Cancer Council
- 1986-present Reviewing Member, Arizona Disease Control Research Commission.
- 1988-1989 Member, Chicago Leukemia Research Society.
- 1988-1989 Member, Small Business Innovation Grant Review Study Section, NCI.
- 1990, 1993 Ad Hoc reviewer, Experimental Therapeutics Study Section 1, NCI.
- 1991, 1992 Ad Hoc reviewer, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
- 1994-1998 Member, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
- 1999-present Advisory Member, "Molecular Targets for therapy of Lung Cancer", National Cancer Institute/CTEP.
- 1999-present Advisory Member, Ohio Cancer Incidence Surveillance System
- 2000 American Cancer Society Immunology and Immunotherapy Study Section, National Division-ad hoc review.

CONSULTANT POSITIONS:

Burrough's Wellcome, 1983-1984

Pfizer Pharmaceutical, 1986-1988

Boehringer Mannheim, 1991-1993

Abbott Laboratories, 1993

Institute for the Study and Treatment of Endometriosis, 1990-present

Adeza Biomedical, 1993

Imutec Corporation, 1993-present

Imutec Corporation; Chairman, Medical Advisory Board, July, 1995-present.

RxKinetic Inc.; Chairman, Medical Scientific Advisory Board. 1997-present.

COMPETITIVE EXTRAMURAL GRANT AWARDS: (note: as Principal Investigator or co-Principal Investigator only)

1. "Cancer Drug Effects on Patient Suppressor Cells". Source: NIH/NCI # CA27598
Period of Support: 09/01/80-03/31/87; as co-Principal Investigator.
2. "Immune Testing in Lung CA During Specific Immunotherapy". Source: NIH/NCI # CA26138. Period of Support: 07/01/80-06/30/83; as co-Principal Investigator.
3. "A Phase I Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2).
Source: NIH/NCI # RFA No-1-CM47667-BRM-MA01. Period of Support: 09/30/84-03/31/87. Subcontract from the Illinois Cancer Council; as Laboratory Principal Investigator for Rush Component.
4. "Phase IB and/or Phase II Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2).
Source: NIH/NCI # RFA No-1 CM47667-03 BRM-MA-04. Period of Support: 09/29/85-02/28/89. Laboratory Principal Investigator for Rush Component.
5. "Arachidonate Metabolism in Cancer Patient Macrophages". Source: NIH/NCI # CA41741. Period of Support: 07/01/88-06/30/92; as Principal Investigator.
6. "LAK Function in Tumor-Infiltrating Leukocytes of Cancer Patients". Source: American Society of Clinical Oncology- 1990 Young Investigator Award to E. Staren, M.D.; as Mentor.
7. "Cancer Patient Macrophage Function in Tumor Environments". Source: NIH/NCI #CA58922. Period of Support: 12/31/92-07/01/96 as Principal Investigator.
8. "American Cancer Society New Investigator Grants in Cancer Research". Source: American Cancer Society. Period of Support: 06/31/95-07/01/97 as Principal Investigator.
9. "Cancer Drug Modulation of Tumor Sensitivity to Macrophages". Source: NIH/NCI, period of support: 12/01/00-11/30/05, as Principal Investigator. status-pending.
10. "Cyclooxygenase Metabolism in Cancer Patient Psychoneuroimmunology". Source: American Cancer Society. Period of Support: 01/01/01-12/31/06, as Principal Investigator. Status-pending.

NONCOMPETITIVE EXTRAMURAL FUNDING: (as principal or co-principal investigator)

1. "The Effect of CGS13080, CGS14854 and CGS53913 on Arachidonic Acid Metabolites and Immune Status of Patients with Solid Tumors". Source: Ciba-Geigy. Period of Support: 10/01/87-09/30/88; as Principal Investigator.

2. "Phase III Protocol for Evaluation of Combined Modalities in the Treatment of Colonic Carcinoma with Positive Nodes, Duke's C, Surgical Resection Alone vs. Postoperative Immunotherapy followed by Chemotherapy". Source: Litton Institutes. Period of Support: 06/30/88-present; as Laboratory Principal Investigator for Rush.
3. "Immunologic Testing and Limited Feldene Administration to Patients with Upper Aerodigestive Tract Squamous Cancer". Source: Pfiser Laboratories, Pfiser Inc. Period of Support: 10/01/89-06/30/91; as Principal Investigator.
4. "Macrophage Function in Women with Endometriosis". Source: Sterling International. Period of Support: 04/01/90-10/01/91; as Principal Investigator for Rush component.
5. "Macrophage Regulation of Endometrial Cell Growth in Women with Endometriosis". Source: Sterling International. Period of Support: 03/01/92-10/01/93; as Principal Investigator for Rush component.
6. "Mechanisms for Modulation of Macrophage Tumoricidal Function in Cancer Patients by Virulizin". Source: Imutec Corporation. Period of Support: 02/01/94-12/01/95; as Principal Investigator.
7. "Immunological Modulation in Pancreatic Cancer Patients treated with Virulizin". Source: Imutec International. Period of Support: 06/30/96-12/31/98; as Principal Investigator.
8. "Modulation of Macrophage Cytolytic Function by Virulizin in Endometriosis". Source: Imutec International. Period of Support: 06/30/96-12/31/97; as Principal Investigator.
9. "Interaction of HIP/PCA particles with leukocytes from Cancer Patients". Source: RxKinetix. Period of Support: 01/01/98 – 08/31/2001; as Principal Investigator.
10. "Endothelin Regulation of Tumor Proliferation and Apoptosis in Human Intracranial Malignancy". Source: Abott Laboratories. Period of Support (pending-to begin in 2000); as Principal Investigator.
11. "Amelioration of TNF α effects in endometriosis by Enbrel". Source: Immunex Corp. Period of support: 01/01/00-06/30/01. As Laboratory Principal Investigator.
11. "H11 binding to human cancer cells". Source: Novopharm Biotechnology. Period of Support: 05/01/00-09/01/01. As Principal Investigator.

HONORS:

USPHS Immunology Trainee, 1973-1974.
USPHA Oncology Trainee, 1974-1976.
Milan V. Novak Award, University of Illinois, Department of Microbiology, 1977.
Who's Who in Cancer Research, 1985
American Men and Women in Science, 1988
1st place award for original research, American Fertility Society, 1992.
Chairman, Poster-Discussion Session, AACR, 1994.
Chairman, Immunology Plenary Session, Vth International Conf. on Endometriosis, 1996.
Chairman, Rationale for Immunotherapy in Endometriosis: VI World Congress on
Endometriosis, Quebec City, Canada, 1998.

ACTIVE MEMBERSHIPS:

American Association for Cancer Research
American Chemical Society
American Association for the Advancement of Science
New York Academy of Science
Society of Biology Response Modifiers
American Fertility Society
American Society of Reproductive Medicine

PUBLICATIONS:

BOOKS EDITED:

1. Prostaglandin Inhibitors in Cancer Immunology and Immunotherapy. eds. JE Harris, DP Braun and KM Anderson. CRC Press, Boca Raton, FLA, 1994.

REVIEWS AND BOOK CHAPTERS:

1. Dray S, and Braun DP: Some perspectives on the transfer of cell mediated immunity by immune RNA. Mol Cell Biochem 25:15, 1979.
2. Braun DP, and Harris JE: Serial immune function testing to predict clinical disease relapse in patients with solid tumors. Cancer Immunol Immunother 15:165, 1983.
3. Harris JE, and Braun DP: The effect of cytotoxic drugs on immunoregulatory cell function in solid tumor cancer patients. Clin Immunol Newsletter 5:113-116, 1984.

4. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. In: Proceedings of the 3rd International Symposium of the Evaluation of the Immunomodifiers, 1984.
5. Braun DP, and Harris JE: Modulation of the immune response by chemotherapy. In: The Modulation of Immunity. Mitchell MS (ed), Oxford: Pergamon Press, 1985.
6. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. Cancer Treat Symp 1:19-26, 1985.
7. Braun DP, and Harris JE: Cancer chemotherapy and its impact on the immune system. In: Fundamentals of Cancer Chemotherapy. Carter SK, and Hellman K (eds), New York: McGraw-Hill, pp 77-97, 1986.
8. Von Roenn J, Harris JE, and Braun DP: Suppressor cell function in solid tumor cancer patients. J Clin Oncol 5:150-159, 1987.
9. Dmowski WP, Braun DP and Gebel H: Endometriosis: Genetic and Immunologic Aspects. in: Current Concepts in Endometriosis. 2nd International Symposium on Endometriosis. Alan R. Liss, Inc. New York, p 99-122, 1989.
10. Dmowski WP, Braun DP and Gebel H: The Immune System in Endometriosis. in Modern Approaches to Endometriosis. J. Rock ed. Kluwer Academic Publishers, p 97-111, 1991.
11. Braun DP and Groenwald SL: The Immune System and Cancer. in Cancer Nursing: Principles and Practice. third edition. Groenwald SL and Goodman M eds. Jones and Bartlett, Boston, MA, pp 70-85, 1993.
12. Harris, J.E. and Braun, D.P.: Tumor Vaccination. in Cancer: Concept to Clinic. Medical Publishing Enterprises. Fair Lawn, NJ; E. Borden, ed. pp. 28-31, 1993.
13. Braun DP: The Impact of Prostaglandins on Cancer Patient Immunity. in Prostaglandin Inhibitors in Tumor Immunology and Immunotherapy. Harris JE, Braun DP and Anderson KM, eds. CRC Press, Boca Raton, Florida, pp. 109-129, 1994.
14. Dmowski WP, Gebel HM and Braun DP. The Role of Cell-Mediated Immunity in Pathogenesis of Endometriosis. Acta Obstet. Gynecol. Scand. Suppl. 73:7-14, 1994.
15. Dmowski WP and Braun DP. Immunological Aspects of Endometriosis. Contemp. Rev. Obstet. Gynaecol. 7: 167-171, 1995.

16. Dmowski WP, Braun DP, and Rotman, C. Aspectos Immunologicos de la endometriosis. in Reproduccion Humana. Remonhi, J, Simon C, Pellicer, A and Bonilla-Musoles, eds. McGraw Hill InterAmericana, Madrid, pp 195-204, 1996.
17. Braun DP and Dmowski WP. Endometriosis: Abnormal Endometrium and Dysfunctional Immune Response. Current Opinion in Obsterics and Gynecology. 10:365-369, 1998.
18. Dmowski WP, Gebel H, and Braun DP. Decreased Apoptosis and sensitivity to macrophage-mediated cytolysis of endometrial cells in endometriosis. Human Reproduction. In Press, 1999.

ARTICLES:

1. Braun DP, and Dray S: Immune RNA mediated transfer of tumor antigen responsiveness to unresponsive peritoneal exudate cells from tumor bearing animals. Cancer Res 37:4138-4144, 1977.
2. Mortensen RF, Braun DP, and Gewurz H: Effects of C-reactive protein on lymphocyte function. III. Inhibition of antigen-induced lymphocyte stimulation and lymphokine production. Cell Immunol 28:59-68, 1977.
3. Braun DP, Hengst J, Moykr M, and Dray S: Antitumor immunity in strain 2 guinea pigs immunized with KCl extracts of L2C tumor cells. J Natl Cancer Inst 60:899-903, 1978.
4. Mokyr M, Braun DP, Usher D, Reiter H, and Dray S: The development of *in vitro* and *in vivo* antitumor cytotoxicity in noncytotoxic, MOPC-315, tumor cells. Cancer Immunol Immunother 4:143-150, 1978.
5. Braun DP, Mokyr M, and Dray S: Generation of anti-MOPC-315 cytotoxicity in uneducated or *in vitro* educated spleen cells from normal or MOPC-315 tumor bearing mice pretreated *in vivo* with BCG. Cancer Res 38:1626-1631, 1978.
6. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in spleen cells of MOPC-315 tumor bearers. In: Cancer Immunology: Experimental and Clinical. Crispin RG (ed), p 211, 1978.
7. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in MOPC-315 tumor bearer spleen cells by depletion of glass adherent cells prior to *in vitro* education. Cancer Res 39:785, 1979.
8. Mokyr M, Bennett JA, Braun DP, Hengst JCD, Mitchell MS, and Dray S: Opposite effects of different strains or batches of the same strain of BCG on the *in vitro*

- generation of syngeneic and allogeneic antitumor cytotoxicity. *J Natl Cancer Inst* 64:339, 1980.
9. Cobleigh MA, Braun DP, and Harris JE: Age dependent changes in human peripheral blood B cell and T cell subsets: Correlation with mitogen responsiveness. *Clin Immunol Immunopathol* 15:162, 1980.
 10. Braun DP, Cobleigh MA, and Harris JE: Selective effect of cytotoxic chemotherapy on immunoregulatory suppressor cells in solid tumor cancer patients. *In: Tumor Progression*. Crispen RG (ed), Philadelphia: Franklin Institute Press, pp 91-102, 1980.
 11. Braun DP, Cobleigh MA, and Harris JE: Multiple concurrent immunoregulatory defects in cancer patients whose peripheral blood leukocytes exhibit depressed PHA induced lympho blastogenesis. *Clin Immunol Immunopathol* 17:89, 1980.
 12. Cobleigh MA, Braun DP, and Harris JE: Quantitation of lymphocytes and T cell subsets (T_G and T_M cells) in disseminated solid tumor cancer patients. *J Natl Cancer Inst* 64:1041, 1980.
 13. Paque RE, Braun DP, and Dray S: Characterization of lymphoid cell RNA which modulates specific cellular immunity. *In: Second International Symposium on RNA in Development*. Academia Sinics, 1980.
 14. Braun DP, and Harris JE: Modulation of immune response by chemotherapy. *Pharmacol Ther* 14:89-122, 1981.
 15. Braun DP, and Harris JE: Effects of combination chemotherapy on immunoregulatory cells in the peripheral blood of solid tumor cancer patients: Correlation with rebound-overshoot immune function recovery. *Clin Immunol Immunopathol* 20:143, 1981.
 16. Braun DP, and Harris JE: Relationship of leukocyte numbers, immunoregulatory cell function and phytohemagglutinin responsiveness in cancer patients. *J Natl Cancer Inst* 67:809, 1981.
 17. Braun DP, Harris JE, Maximovich S, Marder R, and Lint TF: Chemiluminescence in peripheral blood mononuclear cells of solid tumor cancer patients. *Cancer Immunol Immunother* 12:31, 1981.
 18. Braun DP, Penn RD, Flannery AM, and Harris JE: Immunoregulatory cell function in peripheral blood of glioblastoma multiforme patients. *Neurosurg* 12:31, 1981.

19. DeBoer KP, Braun DP, and Harris JE: Natural cytotoxicity and antibody dependent cytotoxicity in solid tumor cancer patients: Regulation by adherent cells. Clin Immunol Immunopathol 23:133, 1982.
20. Harris JE, DeBoer KP, Vahey AL, and Braun DP: The measurement of leukocyte subsets in the peripheral blood of solid tumor cancer patients using monoclonal antibody reagents. Med Pediatr Oncol 10:185, 1982.
21. Taylor SG IV, Saffold P, Braun DP, and Harris JE: T_G cell involvement in the leukocyte adherence inhibition phenomenon. J Natl Cancer Inst 68:549, 1982.
22. Braun DP, DeBoer KP, and Harris JE: Chemiluminescence, suppression and cytotoxicity in peripheral blood mononuclear cells from solid tumor cancer patients. Cancer Immunol Immunother 14:86, 1982.
23. Harris JE, Harris ZL, and Braun DP: Effect of interferon- α on indomethacin sensitive immunoregulatory cells. In: Proceedings of the 15th International Leukocyte Culture Conference. Parker JW, and O'Brien R (eds), Chichester, England: John Wiley and Sons, Ltd., p 635, 1983.
24. Chiu K, Harris JE, Kroin J, Slayton R, and Braun DP: The immunological response of Wistar rats to intracranially implanted C-6 glioma cell line. J Neurooncol 1:365-372, 1983.
25. Penn RD, Kroin JS, Harris JE, Chiu K, and Braun DP: Chronic intratumoral chemotherapy of a rat brain tumor with cisplatin and fluorouracil. Appl Neurophysiol 46:240-244, 1983.
26. Braun DP, Nisius S, Hollinshead AC, and Harris JE: Serial immune testing in surgically resected lung cancer patients. Cancer Immunol Immunother 15:114-121, 1983.
27. Braun DP, Harris ZL, Harris JE, Sandler S, Khandekar J, Locker G, Haid M, Gordon L, Shaw J, Cobleigh MA, and Gallagher P: The effect of interferon therapy on indomethacin sensitive immunoregulation in the peripheral blood mononuclear cells of renal cell carcinoma patients. J Biol Response Mod 2(3):251-262, 1983.
28. Van Epps SF, Stewart LD, Pandey JP, Fudenberg HH, Harris JE, and Braun DP: Immuno-globulin G heavy chain (Gm) allotypes in lung cancer. N Engl J Med 1983.
29. Braun DP, Harris JE, and Rubenstein M: Relationship of arachidonic acid metabolism to indomethacin sensitive immunoregulatory function and lymphocyte PGE sensitivity

- in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. *J Immunopharmacol* 6(3):227-236, 1984.
30. Braun DP, Penn RD, and Harris JE: Regulation of natural killer function by glass adherent cells in patients with primary intracranial malignancies. *Neurosurg* 15(1):29-33, 1984.
 31. Braun DP, and Harris JE: Effects of combination chemotherapy on PGE mediated immunoregulation in the peripheral blood mononuclear cells of solid tumor cancer patients. *J Biol Response Mod* 3:391-396, 1984.
 32. Braun DP, and Harris JE: Abnormal indomethacin sensitive suppression in peripheral blood mononuclear cells of cancer patients restricts augmentation by interleukin 2. *J Biol Response Mod* 3:533-540, 1984.
 33. Chiu KM, McPherson LH, Harris JE, and Braun DP: The separation of cytotoxic human peripheral blood monocytes into high and low phagocytic subsets by centrifugal elutriation. *J Leukocyte Biol* 36:729-737, 1984.
 34. Braun DP, and Harris JE: Effect of cytotoxic antineoplastic chemotherapy on immunoregulatory leukocytes measured with monoclonal antibodies. *Clin Immunol Immunopathol* 33(1):54-56, 1984.
 35. Leslie WT, Templeton A, and Braun DP: Kaposi's sarcoma in the acquired immune deficiency syndrome. *Med Pediatr Oncol* 12:336-342, 1984.
 36. Braun DP, and Harris JE: Effect of chemotherapy on NK function in the peripheral blood of cancer patients. *Cancer Immunol Immunother* 21:240-245, 1986.
 37. Braun DP, and Harris JE: Abnormal monocyte function in patients with Kaposi's sarcoma. *Cancer* 57:1501-1506, 1986.
 38. Janus TJ, Braun DP, and Harris JE: Modulation of lymphocyte responsiveness to phyto-hemagglutinin by micromolecular fibrinogen degradation products. *Clin Immunol Immunopathol* 41:26-34, 1986.
 39. Potempa LA, Gewurz H, Harris JE, and Braun DP: Stimulatory effects of the C-reactive protein subunit on monocyte function, including release of IL 1. *Protides Biol Fluids* 34:287-290, 1986.
 40. Braun DP, Bonomi PD, Taylor SG IV, and Harris JE: Modification of the effects of cytotoxic chemotherapy on the immune responses of cancer patients with a nonsteroidal anti-inflammatory drug, piroxicam. *J Biol Response Mod* 6:331-345, 1987.

41. Harris JE, and Braun DP: Modulation of immune function in cancer patients. Clin Physiol Biochem 5:222-226, 1987.
42. Wiederhold MD, Ondrey F, Anderson KM, Braun DP, and Harris JE: Synthesis of eicosanoids by γ -interferon differentiated U937 cells. Clin Physiol Biochem 6:21-28, 1988.
43. McMannis JD, Fisher RI, Creekmore SP, Braun DP, Harris JE, and Ellis TM: *In vivo* effects of recombinant IL 1. I. Isolation of circulating Leu 19⁺ lymphokine activated killer effector cells from cancer patients receiving recombinant IL 2. J Immunol 140:1335-1340, 1988.
44. Harris JE, and Braun DP: Modulation of *in vitro* antitumor immunity in response to the biological activators of IL 1, γ -interferon, and GM-CSF in the peripheral blood of cancer patients receiving cytotoxic chemotherapy. Ann NY Acad Med 65:16-25, 1988.
45. Braun DP, Kessler H, Falk L, Paul D, Harris JE, Blaauw B, and Landay AL: Monocyte functional studies in asymptomatic HIV-infected individuals. J Clin Immunol 8:486-494, 1988.
46. Staren ED, Braun DP, Harris JE, Bines SD, and Economou SG: Lymphokine activated killer cell induction in the peripheral blood mononuclear cells of colon cancer patients. Surg Forum 39:447-449, 1988.
47. Ellis TM, Creekmore S, McMannis J, Braun DP, Harris JE, and Fisher RI: Appearance and phenotypic characterization of circulating Leu 19⁺ cells in cancer patients receiving recombinant interleukin 2. Cancer Res 48:6597-6602, 1988.
48. Braun DP, Taylor ST IV, and Harris JE: Modulation of immunity in cancer patients by prostaglandin antagonists. Prog Clin Biol Res 288:444-448, 1989.
49. Creekmore SP, Harris JE, Ellis TM, Braun DP, Cohen II, Bhoopalam N, Jassak PF, Cahill MA, Canzoneri CL, and Fisher RI: A phase I clinical trial of recombinant interleukin 2 by periodic 24 hour infusions. J Clin Oncol 7:276-284, 1989.
50. Staren ED, Economou SG, Harris JE, and Braun DP: Lymphokine activated killer cell induction in tumor infiltrating leukocytes from colon cancer patients. Cancer 64:2238-2242, 1989.
51. Braun DP, Siziopikou KP, Casey LC, and Harris JE: The *in vitro* development of cytotoxicity in response to granulocyte/macrophage colony stimulating factor or γ -interferon in the peripheral blood monocytes of patients with solid tumors. Modulation

- by arachidonic acid metabolic inhibitors. *Cancer Immunol Immunother.* 32:55-61, 1990.
52. Harris JE, and Braun DP: The effect of aspirin and other cyclooxygenase inhibitors on antitumor immunity. In: *Aspirin towards 2000* (G. Fryers, editor). pp. 45-52. Royal Society of Medicine. 1990.
53. Siziopikou KP, Harris JE, Casey L, Nawans Y and Braun DP. Impaired Tumoricidal Function of Alveolar Macrophages from Patients with Non-small Cell Lung Cancer. *Cancer*, 68:1035-1044, 1991.
54. Braun, D.P., Gebel, H., Rana, N, Rotman, C, and Dmowski, W.P. Peritoneal Macrophage Function in Women with Endometriosis., *Fertility and Sterility* 57: 1203-1210, 1992.
55. Harris, J.E. and Braun, D.P.: Abnormal immunoregulation in human cancer at the actual tumor site. In "Combination Therapies. Biological Response Modifiers in the Treatment of Cancer and Infectious Diseases. A. Goldstein, ed. Plenum Publishing, New York, pp. 197-206, 1992.
56. Harris, J.E. and Braun, D.P.: Abnormal immunoregulation and the tumor dormant state in human cancer. In "Cellular Immune Mechanisms and Tumor Dormancy" (T. Stewart, editor). CRC Press, Boca Raton, FLA, pp. 261-280, 1992.
57. Chu, E., Casey, L.C., Harris, J.E. and Braun, D.P. Suppression of the Development of Tumoricidal Function in Gamma Interferon-treated Human Peripheral Blood Monocytes by Lipopolysaccharide: The role of Cyclooxygenase Metabolites. *J. of Clin. Immunol.* 13: 49-57, 1993.
58. Braun, DP, Ahn, M, Chu, E, Casey, L.C., Harris, JE, Wilbanks, G, and Siziopikou, KP. The sensitivity of macrophages from Different Anatomical Sites of Cancer Patients to Arachidonic Acid Metabolism. *Cancer Research* 53: 3362-3368, 1993.
59. Braun, DP, Muriana M, Gebel H, Rotman C, Rana N and Dmowski WP. Monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. *Fertility and Sterility* 61: 78-85, 1994.
60. Braun, DP, Gebel H and Dmowski WP. Effect of danazol in vitro and in vivo on monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. *Fertility and Sterility* 62: 89-95, 1994.

61. Gebel HM, Braun DP, Rotman C, Rana N and Dmowski WP. Decreased in vitro production of IgG in women with severe endometriosis. *Amer. J. Reprod. Immunol.* 29: 124-130, 1993.
62. Dmowski WP, Gebel HM and Braun DP. The role of cell-mediated immunity in pathogenesis of endometriosis. *Acta Obstet Gynecol Scand* 159: 7-14, 1994.
63. Gebel HM, Rana N, Braun DP, and Dmowski WP. Differential Expression of VLAB1 (CD29) on monocytes from patients with endometriosis. *Amer J Reprod Immunol* 34: 317-322, 1995.
64. Rana N, Braun DP, Rotman C, Gebel HM and Dmowski WP. Cytokine Synthesis by peritoneal macrophages in patients with endometriosis. *Fertility Sterility*, 65:925-931, 1996.
65. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Cytokine synthesis by peripheral blood monocytes of patients with endometriosis. *Fertility and Sterility* 65: 1125-1129, 1996.
66. Braun DP and Preisler HD. Cytolytic Activity of Peripheral Blood Blast Cells from Patients with Acute Myeloid Leukemia. *Leukemia and Lymphoma* 27: 459-467, 1997.
67. Siziopikou KP, Ahn M, Casey LC, Silver M, and Braun DP. Augmentation of impaired tumoricidal function in alveolar macrophages from lung cancer patients by coculture with allogeneic lymphocytes. *Cancer Immunol. Immunother.* 45:29-36, 1997.
68. Ahn M, Siziopikou KP, Casey LC, Harris JE and Braun DP. Suppression of tumoricidal function in alveolar macrophages from lung cancer patients by Interleukin-6. *Cancer Immunol. Immunother.* 45: 37-44, 1997.
69. Ono N, Glick R, Kroin J. Penn RD and Braun DP. Effect of IGF inhibitor on proliferation of rat C6 glioma and human glioma explants. Submitted to *J. Neurosurgery*.
70. Braun DP, Gebel HG, Rana N, and Dmowski WP. Cytolysis of eutopic and ectopic endometrial cells by peripheral blood monocytes and peritoneal macrophages in women with endometriosis. *Fertility and Sterility* 69: 1103-, 1998.
71. Gebel HG, Braun DP, Frame D, Tambur A, Rana N and Dmowski WP. Spontaneous Apoptosis in eutopic and ectopic endometrium from women with endometriosis. *Fertility and Sterility* 69: 1042-1047, 1998.

72. Wong PY, Staren ED, Tereshkova N, and Braun DP: Functional Analysis of Tumor-infiltrating leukocytes in breast cancer patients. J. Surg. Res. 76: 95-103, 1998
73. Wilbanks GD, Ahn M, Beck DA and Braun DP. Antitumor cytotoxicity of peripheral blood monocytes and peritoneal macrophages from patients with gynecological malignancies. Int. J. Gynecological Cancer 9: 427-432, 1999.
74. Ferdinandi E, Braun DP, Liu C, Zee B, and Ely G. Virulizin: A Review of its Antineoplastic Activity. Exp Opin. Invest. Drugs 8: 1721-1735, 1999.
75. Ortel JW, Staren ED, Faber LP, Warren WH and Braun, DP. Cytokine Biosynthesis by Tumor-Infiltrating T Lymphocytes from Human Non-Small Cell Lung Carcinoma. Cancer Immunology and Immunotherapy, 48:627, 2000.
76. Ortel JW, Staren ED, Faber LP, Warren WH and Braun, DP. Modulation of Autologous Tumor cytotoxicity by Tumor-infiltrating Lymphocytes from non-Small Cell Lung Carcinoma by Type 2 Cytokines and Transforming Growth Factor- β . Submitted to Journal of Cytokine Research, 2000.
77. Dmowski, WP, Ding, J, Shen, J, Rana, N, Fernandez, BB, Braun, DP. Apoptosis in Endometrial Glandular and Stromal Cells in Women With and Without Endometriosis. Submitted to Human Reproduction, July 2000.

ABSTRACTS AND LETTERS:

1. Braun DP, and Dray S: Tumor immune RNA conversion of lymphoid cells of tumor-bearing mice from being unresponsive to responsive upon challenge with tumor specific antigen. Cancer Res 17:7, 1976.
2. Braun DP, and Dray S: The effect of BCG on the *in vitro* education of normal spleen cells and of tumor bearing spleen cells. Cancer Res 19:154, 1978.
3. Hengst JCD, and Braun DP: Opposite effects of different batches of BCG on the *in vitro* generation of antitumor cytotoxicity. Cancer Res 20:67, 1979.
4. Braun DP, Cobleigh MA, and Harris JE: Explanation for rebound-overshoot recovery of immune function in cancer patients receiving cytotoxic drugs. Clin Res 27:643A, 1979.
5. Cobleigh MA, Braun DP, and Harris JE: Lymphocyte subset variation in normal aging subjects and in cancer patients. Clin Res 27:643A, 1979.

6. Cobleigh MA, Braun DP, and Harris JE: Multiple concurrent suppressor cell mechanisms in human malignant disease. Blood 54(1):97A, 1979.
7. Braun DP, Cobleigh MA, and Harris JE: The rebound-overshoot phenomenon: A consequence of selective cytotoxic drug effect on immunoregulatory suppressor mechanisms. Proc Am Assoc Cancer Res 21:373, 1980.
8. Braun DP, Rossof AH, and Harris JE: Relationship of monocyte percentage and monocyte suppressor activity to T lymphocyte function in solid tumor cancer patients. Proc Am Assoc Cancer Res 21:370, 1981.
9. Harris JE, and Braun DP: Effects of adjuvant chemotherapy for stage II breast cancer on immunoregulatory cell function. Proc Am Assoc Cancer Res 21:370, 1981.
10. Harris JE, and Braun DP: Effects of combination chemotherapy on immunoregulatory cell functions in solid tumor cancer patients: Correlation with rebound-overshoot recovery of immunity. Proc Am Assoc Cancer Res 21:183, 1981.
11. Braun DP, and Harris JE: Monocyte suppression and activation in solid tumor cancer patients. Proc Am Assoc Cancer Res 21:185, 1981.
12. Chiu KM, Braun DP, and Harris JE: Suppression of alloimmune response in normal individuals and cancer patients by indomethacin sensitive cells. Clin Res 29:735A, 1981.
13. DeBoer KP, Braun DP, and Harris JE: Adherent cell suppression of natural cytotoxicity in solid tumor patients. Clin Res 29:735A, 1981.
14. Rubenstein M, Braun DP, and Harris JE: Abnormal prostaglandin metabolism in cancer patient monocytes. Clin Res 29:737A, 1981.
15. Braun DP, DeBoer KP, and Harris JE: Relationship of monocyte prostaglandin metabolism and monocyte indomethacin sensitive immunoregulatory function in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. Blood 58:70a, 1981.
16. Harris JE, Braun DP, DeBoer KP, and Vahey AL: Enumeration with monoclonal antibodies of leukocyte subsets in peripheral blood of solid tumor cancer patients. Blood 58:83a, 1981.
17. Harris JE, Vahey AL, and Braun DP: Quantitation with monoclonal antibodies of leukocyte subsets in peripheral blood mononuclear cells of solid tumor cancer patients. Proc Am Soc Clin Oncol 1:42, 1982.

18. Chiu KM, Droin J, Harris JE, and Braun DP: The immunological response of Wistar rats to the C-6 glioma cell line. *Proc Am Assoc Cancer Res* 23:241, 1982.
19. Harris JE, and Braun DP: Suppressor cell development predicts for tumor recurrence in surgically resected lung cancer. *Proc Am Assoc Cancer Res* 23:250, 1982.
20. Penn RD, Showel J, Harris JE, Braun DP, and Wolter J: Immunoregulatory cell studies in patients with gliomas. *Proc Am Assoc Cancer Res* 23:251, 1982.
21. Braun DP, and Harris JE: Relationship of arachidonic acid metabolism and indomethacin sensitive immunoregulatory function in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. *Proc Am Assoc Cancer Res* 23:262, 1982.
22. Harris ZL, Braun DP, and Harris JE: Interferon therapy causes impaired lymphoproliferation by prostaglandin mediated mechanisms. *Clin Res* 30(1):747A, 1982.
23. Harris JE, and Braun DP: The effect of interferon therapy on immunoregulatory cells in the peripheral blood of patients with renal cell carcinoma. *Immunobiol* 163:255, 1982.
24. Braun DP, and Harris JE: Effect of chemotherapy on immunoregulation in solid tumor cancer patients: Correlation with changes in monocyte PGE synthesis and lymphocyte PGE sensitivity. *Proc Am Assoc Cancer Res* 24:215, 1983.
25. Harris JE, Harris ZL, and Braun DP: Depression of monocyte induced lymphoproliferative responses in peripheral blood mononuclear cells (PBMC) from renal cell carcinoma patients treated with human leukocyte interferon (IFN- α). *Proc Am Assoc Cancer Res* 24:205, 1983.
26. Chiu KM, McPherson LH, Harris JE, Slayton RE, and Braun DP: The separation of cytotoxic human peripheral blood monocytes into phagocytic and nonphagocytic subsets by centrifugal elutriation. *Proc Am Assoc Cancer Res* 24:215, 1983.
27. Braun DP, Harris JE, and Taylor SG IV: Effect of chemotherapy on monoclonal antibody defined leukocyte subsets in solid tumor cancer patients. *Proc Am Soc Clin Oncol* 2:56, 1983.
28. Penn RD, Braun DP, Harris JE, and Rossof AH: Modulation of natural killer cell function in brain tumor patients by glass adherent suppressor cells. *Proc Am Soc Clin Oncol* 3:57, 1984.

29. Braun DP, Harris JE, and Taylor SG III: Effect of chemotherapy on monoclonal antibody defined leukocyte subsets in solid tumor cancer patients. Proc Am Assoc Cancer Res 25:234, 1984.
30. Braun DP, and Harris JE: Chemotherapeutic modulation of NK function in peripheral blood mononuclear cells of cancer patients. Proc Am Assoc Cancer Res 25:234, 1983.
31. Braun DP, Wolter J, and Harris JE: NK function and immunoregulation in peripheral blood mononuclear cells (PBMC) from sarcoma patients treated with fibroblast interferon. Proc Am Soc Clin Oncol 3:56, 1984.
32. Janus T, Slayton WB, Harris JE, Rossof AH, and Braun DP: Modulation of lymphocyte responsiveness to phytohemagglutinin (PHA) by micromolecular fibrinogen degradation products (FDP) due to induction of monocyte suppressor cells. Proc Am Assoc Cancer Res 25:243, 1984.
33. Harris JE, Wiederhold MD, Slayton J, and Braun DP: Abnormal arachidonate conversion to prostaglandins, leukotrienes and eicosatetraenoic acids in cancer patient monocytes. Blood 64:190a, 1984.
34. Wiederhold MD, Harris JE, and Braun DP: Modulation of arachidonate incorporation into macrophage membrane phospholipids by interferon. Fed Proc 44:951, 1985.
35. Braun DP, Von Roenn J, and Harris JE: Modification of chemotherapy induced immunosuppression in lung cancer patients with piroxicam. Proc Am Soc Clin Oncol 4:223, 1985.
36. Taylor SG IV, Harris JE, and Braun DP: Potentiation of T cell immunity in head and neck cancer patients with nonsteroidal antiinflammatory agents. Proc Am Assoc Cancer Res 26:278, 1985.
37. Leslie WT, Harris JE, Slayton RE, Wiederhold MD, Slayton JM, and Braun DP: Modulation of arachidonic acid metabolism in monocytes by α - and γ -interferon. Proc Am Assoc Cancer Res 26:279, 1985.
38. Harris JE, and Braun DP: Abnormal monocyte function in patients with Kaposi's sarcoma. Proc Am Assoc Cancer Res 26:286, 1985.
39. Anderson KM, Harris JE, Saffold P, and Braun DP: Modulation of cyclic nucleotide responses in lymphocytes by prostaglandin and interleukin 2. Proc Am Assoc Cancer Res 26:303, 1985.

40. Wiederhold MD, Slayton JM, Harris JE, Anderson KM, and Braun DP: Stimulation of phospholipase activity in U937 cells following treatment with α - and γ -interferon. Proc Am Assoc Cancer Res 26:307, 1985.
41. Williams GS, Harris JE, and Braun DP: The role of arachidonic acid metabolism in spontaneous cytotoxicity and immunoregulation of subsets of human peripheral blood monocytes. Proc Am Assoc Cancer Res 26:307, 1985.
42. Braun DP, Harris JE, Slayton JM, and Wiederhold MD: Altered arachidonic acid metabolism in cancer patient monocytes. Proc Am Assoc Cancer Res 26:307, 1985.
43. Harris JE, Wiederhold MD, Slayton JM, and Braun DP: Arachidonate incorporation and release from the membrane phospholipids of cancer patient monocytes. J Leukocyte Biol 38:133, 1985.
44. Williams GS, Harris JE, and Braun DP: Differences in accessory cell function of subsets of human peripheral blood monocytes. J Leukocyte Biol 38:180, 1985.
45. Harris JE, Janus TJ, and Braun DP: Fibrinogen degradation products (FDP) induce normal monocytes to become suppressor cells by modifying arachidonic acid metabolism. Blood 66:87a, 1985.
46. Ondrey F, Wiederhold MD, Anderson KM, Braun DP, and Harris JE: Early PMA-induced morphologic changes, release of arachidonic acid, and synthesis of eicosanoids in U937 cells. Proc Am Assoc Cancer Res 27:58, 1986.
47. Leslie WT, Taylor SG IV, Harris JE, and Braun DP: Immune studies in renal cell carcinoma (RCC) patients treated with interferon. Proc Am Assoc Cancer Res 27:321, 1986.
48. Harris JE, and Braun DP: Modification of chemotherapy induced immunosuppression by the nonsteroidal antiinflammatory drug (NSAID), piroxicam. Proc Am Assoc Cancer Res 27:328, 1986.
49. Braun DP, Roseman D, and Harris JE: Enhanced interleukin 1 (IL 1) synthesis by monocytes from colon cancer patients. Proc Am Assoc Cancer Res 27:345, 1986.
50. Ebie N, Harris JE, Anderson KM, Wolter J, and Braun DP: Cyclic nucleotide responses in peripheral blood mononuclear cells of cancer patients following stimulation with interleukin 2 (IL 2) or prostaglandin E (PGE). Proc Am Assoc Cancer Res 27:345, 1986.

51. Wiederhold MD, Anderson KM, Ondrey F, Braun DP, and Harris JE: Analysis of monocyte cellular phospholipid fatty acid composition in patients with renal cell carcinoma with and without lymphoblastoid interferon (Hu IFN Ly) therapy. Proc Am Assoc Cancer Res 27:349, 1986.
52. Williams GS, Harris JE, Rossof AH, and Braun DP: Arachidonic acid (AA) metabolism differences in subsets of human peripheral blood monocytes separated by elutriation (CCE). Proc Am Assoc Cancer Res 27:350, 1986.
53. Harris JE, Landay AL, Falk L, Paul D, and Braun DP: Monocyte functional disturbances in asymptomatic homosexual males (AHM), AIDS-related complex syndrome (ARCS) patients, and acquired immune deficiency patients (AIDS). Blood 68:127a, 1986.
54. Landay AL, Kessler H, Braun DP, Harris JE, Paul D, and Falk L: Isolation of human immunodeficiency virus (HIV) from peripheral blood monocytes of subjects at risk for AIDS. Blood 68:129a, 1986.
55. Siebert P, Wiederhold MD, Anderson KM, Ondrey F, Harris JE, and Braun DP: Morphologic changes and alterations in eicosanoid metabolism induced in U937 cells by γ -interferon. Proc Am Assoc Cancer Res 28:339, #1341, 1987.
56. Conlon K, Harris JE, and Braun DP: Modulation of lymphokine activated killer (LAK) cell induction with eicosanoid synthesis inhibitors. Proc Am Assoc Cancer Res 28:367, #1455, 1987.
57. Chu EB, Potempa LA, Harris JE, Gewurz H, and Braun DP: Modulation of monocyte cytotoxicity with urea-chelated and acid-modified C-reactive protein (CRP). Proc Am Assoc Cancer Res 28:344, #1362, 1987.
58. Braun DP, Staren ED, and Harris JE: Lymphokine activated killer cell (LAK) induction in tumor infiltrating leukocytes (TIL) and peripheral blood mononuclear cells (PBMC) from colon cancer patients. Proc Am Assoc Cancer Res 28:371, #1472, 1987.
59. Potempa LA, Gewurz H, Harris JE, and Braun DP: Modulation of monocyte function by modified antigenically distinct forms of C-reactive protein (CRP). Proc Am Assoc Cancer Res 28:344, #1363, 1987.
60. Harris JE, Taylor SG IV, and Braun DP: Modulation of lymphokine activated killer (LAK) cell induction following cytotoxic chemotherapy. Proc Am Assoc Cancer Res 28:372, #1475, 1987.

61. Creekmore SP, Harris JE, Ellis TM, Braun DP, McMannis JD, Cohen II, Bhoopalam N, Jassak PF, Cahill MA, Canzoneri CL, and Fisher RI: Phase I/II trial of recombinant interleukin 2 by 24 hour continuous infusion. An Illinois Cancer Council Trial. Proc Am Soc Clin Oncol 6:244, #960, 1987.
62. Harris JE, Landay AL, Kessler H, Falk L, Paul D, and Braun DP: Abnormal indomethacin sensitive suppressor cell (ISSC) function is associated with deficient lymphokine activated killer (LAK) cell induction in patients infected with the human immunodeficiency virus (HIV). Proc Am Soc Clin Oncol 6:3, #11, 1987.
63. Ellis T, Braun DP, Creekmore SP, Bhoopalam N, Harris JE, and Fisher RI: Appearance and phenotypic characterization of circulating Leu 19⁺ cells in patients receiving recombinant IL 2. Proc Am Assoc Cancer Res 28:373, #1480, 1987.
64. McMannis JD, Braun DP, Fisher RI, Creekmore SP, Harris JE, and Ellis TM: Demonstration of circulating lymphokine activated killer (LAK) cells in patients receiving interleukin 2 (IL 2). Proc Am Assoc Cancer Res 28:372, #1474, 1987.
65. Landay AL, Harris JE, Falk L, Paul D, Kessler H, and Braun DP: Abnormal lymphokine activated killer cell (LAK) induction in peripheral blood mononuclear cells (PBMC) in patients infected with the human immunodeficiency virus (HIV). Proc 3rd International Conference on AIDS, 1987.
66. Levy RA, Harris JE, Bagdade JD, and Braun DP: Marine lipids suppress monocyte mediated cytotoxicity in women with insulin dependent diabetes mellitus. Clin Res 35(6):826A, 1987.
67. Braun DP, and Harris JE: Improved response to monocyte activators in the peripheral blood monocytes of patients with solid tumors following cytotoxic chemotherapy. Proc Am Assoc Cancer Res 29:373, #1484, 1988.
68. Chu EB, Potempa LA, Harris JE, Gewurz H, and Braun DP: Reversal of Neo-C-reactive protein mediated suppression of monocyte cytotoxicity by modulation of arachidonate metabolism. Proc Am Assoc Cancer Res 29:371, #1477, 1988.
69. Staren ED, Braun DP, Harris JE, and Economou SG: Lymphokine activated killer cell induction in tumor infiltrating leukocytes and peripheral blood mononuclear cells from colon cancer patients. Proc Am Assoc Cancer Res 29:406, #1617, 1988.
70. Taylor SG IV, Harris JE, and Braun DP: Modulation of monocyte cytotoxicity by arachidonic acid metabolic inhibitors in the peripheral blood monocytes of patients with solid tumors. Proc Am Assoc Cancer Res 29:371, #1478, 1988.

71. Staren ED, Harris JE, and Braun DP: Lymphokine activated killer (LAK) cell induction in the peripheral blood mononuclear cells (PBMC) of colon cancer patients. Proc Am Soc Clin Oncol 7:98, #373, 1988.
72. Braun DP, Siziopikou KP, and Harris JE: Modulation of monocyte cytotoxicity in response to granulocyte/macrophage colony stimulating factor (GM-CSF) and γ -interferon (γ -IFN) by arachidonic acid metabolic inhibitors. Blood 72:142a, 1988.
73. Gaynor ER, Ellis TM, Harris JE, Braun DP, Tuteur D, Creekmore S, and Fisher RI: Adjuvant immunotherapy using intermittent 24 hour infusion of recombinant interleukin 2 (rIL 2) in patients with surgically resected colon carcinoma at high risk for recurrence. Proc Am Soc Clin Oncol 8:190, #739, 1989.
74. Braun DP, Harris JE, Landay AL, and Kessler H: Impaired development of monocyte tumor cytotoxicity in response to γ -interferon (γ -IFN) and granulocyte/macrophage colony stimulating factor (GM-CSF) in the peripheral blood monocytes of asymptomatic human immunodeficiency virus (HIV) infected patients. Potentiation of responsiveness by indomethacin. Proc Am Soc Clin Oncol 8:1, #4, 1989.
75. Staren ED, Braun DP, Harris JE, Bines SD, and Economou SG: Characterization of interleukin 2 (IL 2) activated killer cells in tumor infiltrating leukocytes (TIL) from colon cancer patients. Proc Am Soc Clin Oncol 8:103, #398, 1989.
76. Siziopikou KP, Casey LC, Harris JE, and Braun DP: Impaired development of tumoricidal function in the alveolar macrophages (AM) of patients with lung cancer. Proc Am Assoc Cancer Res 30:334, #1323, 1989.
77. Chu EB, Potempa LA, Gewurz H, Harris JE, and Braun DP: Modulation of the development of tumoricidal function in response to macrophage activators by a modified form of the C-reactive protein. Proc Am Assoc Cancer Res 30:333, #1322, 1989.
78. Braun DP, Siziopikou KP, and Harris JE: Modulation of the *in vitro* development of tumoricidal function in response to combinations of macrophage activators in the peripheral blood monocytes (PBM) of cancer patients (pts). Proc Am Assoc Cancer Res 30:334, #1324.
79. Taylor SG IV, Harlan S, McHale M, Rudolph A, Braun DP, and Harris JE: Phase I dose escalation of TNF/IL 2 combination therapy in human malignancies. Proc Am Assoc Cancer Res 30:405, #1609, 1989.

80. Staren ED, Economou SG, Harris JE, Bines S, and Braun DP: Tumor induced modulation of lymphokine activated killer cell induction in tumor infiltrating leukocytes. Proc Am Assoc Cancer Res 31:238, #1405, 1990.
81. Braun DP, Siziopikou KP, Casey L, and Harris JE: Augmentation of impaired tumoricidal function in alveolar macrophages from lung cancer patients by cocultivation with allogeneic lymphocytes. Proc Am Assoc Cancer Res 31:244, #1443, 1990.
82. Siziopikou KP, Casey L, Harris JE, and Braun DP: Tumor necrosis factor secretion, interleukin 1 secretion, and the development of cytotoxicity in alveolar macrophages from lung cancer patients. Proc Am Assoc Cancer Res 31:244, #1442, 1990.
83. Harris, JE, Siziopikou KP, Ahn M, Wilbanks GD, Jordan E and Braun DP: The induction of tumoricidal function in regional versus systemic macrophages from patients with solid tumors. Proc Am Assoc Clin Oncol, 10:214, 1991.
84. Chu EB, Gewurz H, Harris JE and Braun DP: Arachidonic acid metabolism in macrophages treated with a modified form of the C-reactive protein. Proc Am Assoc Cancer Res, 32:232, 1991.
85. Braun DP, Siziopikou KP, Ahn M and Harris JE: Induction of tumoricidal function in macrophages from cancer patients by anti-CD3 monoclonal antibodies. Proc Am Assoc Cancer Res, 32:232, 1991.
86. Harris JE, Siziopikou KP, Casey LM and Braun DP: Sensitivity of alveolar macrophages and peripheral blood monocytes to modulation of arachidonic acid metabolism. Proc Am Assoc Cancer Res, 32:233, 1991.
87. Harris JE, Ahn M and Braun DP: Differential sensitivity of peripheral blood monocytes (PBM), alveolar macrophages (AM) and peritoneal macrophages (PM) from cancer patients to modulation of arachidonic acid (AA) metabolism. Blood 78:1072, 1991.
88. Harris JE and Braun DP: Natural Killer (NK) cell function in renal cell cancer patients (RCC pts): Identification of a subset responsive to immunological manipulation. Proc. Am. Soc. Clin. Oncol. 11:257, 1992.
89. Braun DP, Ahn M, and Harris JE: Sensitivity of human macrophages from different anatomical sites to arachidonic acid metabolism. Proc. Am. Can. Res. 33:305, 1992.
90. Ahn M, Harris JE, Wilbanks GW, and Braun DP: Induction of tumoricidal function in macrophages by anti-CD3 monoclonal antibodies. Proc. Am. Assoc. Can. Res. 33:305, 1992.

91. Ahn M, Plate JMD, Harris JE, and Braun DP: Downregulation of Interleukin 6 in alveolar macrophages of lung cancer patients facilitates the development of tumoricidal function. *Blood* 80:103a, 1992.
92. Braun DP, Ahn M, Harris JE, Wilbanks G, and Plate J: Altered synthesis of cytokine messenger RNA (m RNA) in peripheral blood monocytes and peritoneal macrophages from patients with gynecological malignancies. *Proc. Am. Soc. Clin. Oncol.* 12:299, 1993.
93. Ahn M, Plate J, Harris JE, and Braun DP: Allogeneic lymphocytes stimulate macrophage tumoricidal function in association with IL-6 suppression. *Proc. Am. Assoc. Cancer Res.* 34:445, 1993.
94. Harris JE, Ahn M, and Braun DP: Interleukin-12 (IL-12) is superior to gamma interferon (IFN γ) plus endotoxin and to Monocyte Colony Stimulating Factor (M-CSF) in stimulating tumoricidal function in alveolar macrophages (AM) from patients with non-small cell lung cancer. *Blood*, 82:509a, 1993.
95. Taylor SG IV, Braun DP, Coon JS, Hutchinson JC, Panje WR, Caldarelli DD, Shott S and Preisler HD. Augmentation of autologous tumoricidal function in peripheral blood monocytes from head and neck cancer patients by treatment with indomethacin. *Proceedings American Association for Cancer Research* 35: 480, 1994.
96. Braun DP, Coon JS, Taylor SG IV, Hutchinson JC, Panje WR, Caldarelli DD, Shott S and Preisler HD. Discordance of leukocyte-mediated tumoricidal function in the blood and leukocyte infiltration in tumor tissues. *Proceedings American Association for Cancer Research* 35: 494, 1994.
97. Effect of endometriosis on the production of cytokines by peripheral blood monocytes. D.P. Braun, N. Rana, C. Rotman, H.M. Gebel and W.P. Dmowski. *Proc. Amer. Fertil. Soc.* 50: s20, 1994.
98. Effect of danazol (DZ) or gonadotropin releasing hormone agonist (GnRH-A) therapy on the production of cytokines by peripheral blood monocytes (PBM) from patients with endometriosis (ENDO). D.P. Braun, N. Rana, C. Rotman, H.M. Gebel and W.P. Dmowski. *Proc. Amer. Fertil. Soc.* 50: 149, 1994.
99. Braun DP, Tereshkova N, Ortegell J and Preisler HD. Cytotoxic function of peripheral blood blast cells from patients with acute myelogenous leukemia. *Proceedings American Association for Cancer Research* 36: 473, 1995.

100. Braun, DP, Taylor, SGT IV, Coon, J, Griem, K. LaFollette, S. Hutchinson, J, Kotelnikov, V, and Preisler, HP. Biological and Immunological Differences in Primary and Recurrent Head and Neck Cancer. Proc. Amer.Soc.of Clin. Oncol. 14:22,1995.
101. Taylor SGT IV, Braun DP, Kotelnikov VM, Wood NB, Mundle S, Coon J, Raza A, LaFollette S, Griem K, Hutchinson J, Cladarelli D, Panje W, Friedman M, Preisler H. Tumor proliferative and immunologic alterations during combined chemotherapy/radiation in Head and Neck Cancer. Proc. ASCO. 15:, 315, 1996.
102. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Differential Sensitivity of endometrial cells from uterus and peritoneal implants to macrophage-mediated lysis in patients with endometriosis. Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.
103. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Macrophage functional changes in women with unexplained infertility identifies a subset of patients with subclinical endometriosis. Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.
104. DmowskiWP, Rana N, Braun DP, Gebel HG, and House R. Is there a pathophysiological basis for generalized symptoms in women with endometriosis? Proceedings of the Vth World Congress on Endometriosis. Yokohama, Japan. October, 1996.
105. Braun DP, Gebel HG, Rana N, and Dmowski WP. Cytolysis of endometrial cells (EC) by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) in women with endometriosis (Endo). Proceedings of the 52nd Annual Meeting, American Society of Reproductive Medicine, 1996.
106. Braun DP, Harris JE, Gebel H, Plate J, Deziel D, Millikan K, Doolas A and Prinz R. Immunologic Evaluation of pancreatic cancer patients. Proc. Amer. Assoc. Cancer Res. 38:487, 1997.
107. Ortegell J, Staren E, Faber P, Warren W, and Braun D. Cytokine biosynthesis by tumor infiltrating T cells in non-small cell lung cancers. Proc. Amer. Assoc. Cancer Res. 38:630, 1997.
108. Braun, DP, Dmowski, WP. Stimulation of eutopic and ectopic endometrial cell proliferation by autologous peritoneal fluids with endometriosis is due to tumor necrosis factor-alpha (TNF α). Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontario, Canada, September 25-30,1999.

109. Ding, J, Shen, J, Braun, DP, Rana, N, Fernandez, BB, Dmowski, WP. Apoptosis in Endometrial Glandular and Stromal Cells in Women with and without Endometriosis. Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontario, Canada, September 25-30, 1999.
110. Shen, J., Ding, J, Braun, DP, Rana, N, Dowbrowski, A, Dmowski, WP. Endometrial Macrophages May Be Involved in the Regulation of Endometrial Apoptosis. Poster Presentation, ASRM/CFAS '99 Conjoint Annual meeting, Ontario, Canada, September 25-30, 1999.
111. Ding, J, Shen, M, Gogacz, M, Braun, DP, Rana, N., Dmowski, WP. Endometrial Apoptosis Is Inhibited In Vitro by TNF α in Women with Endometriosis and Is Stimulated by TNF α in Healthy Women. Journal of the Society for Gynecologic Investigation, 2000.
112. Braun, DP, Ding, J, Shen, M, Gogacz, M., Dmowski, WP. Modulation of Endometrial Cell Apoptosis and Proliferation by Tumor Necrosis Factor in Women with Endometriosis. Journal of the Society for Gynecologic Investigation, 2000.
113. Ding, J, Gogacz, Shen, M, Braun, DP, Rana, N, Dmowski. Expression of TNF α Receptor Type-I Gene in Endometrial Cells from Women with and without Endometriosis. Submitted to American Society For Reproductive Medicine, 56th Annual Meeting, October 21-25, 2000, San Diego, California.

UNIVERSITY INSTRUCTION:

Course Director

Principles of Immunology. University of Illinois, Dept. of Microbiology, 1977.

Biology of Cancer. College of Nursing, Rush Presbyterian St. Luke's Medical Center, 1982.

Tumor Immunology. Department of Immunology, College of Medicine, Rush Presbyterian St. Luke's Medical Center, 1985.

The Basic Science of Oncology. Rush Cancer Institute. 1993-1995.

Lecturer

Basic and Clinical Immunology, Department of Immunology/Microbiology
Cellular Immunology, Department of Immunology/Microbiology

Tumor Immunology, Department of Nursing
Pathophysiology of Malignant Disease, Department of Pathology
Medical Oncology Didactic Course

Organizing

Medical Oncology Didactic Lecture Series
Medical Oncology Research Seminar Series
Oncology Fellow Basic Sciences Journal Club

Advisory

Graduate Students Matriculated (5)
Dissertation Advisory Committees (18)
 As Chairman (2)
 As Advisor (5)
 As Member (11)
 Other Department (2)
 Other University (5; 4, University of Illinois, 1 Notre Dame)

UNIVERSITY COMMITTEES:

Student Judiciary Review
Academic Freedom, Chairman, 1994-1996
CED Review of Rush Medical College
Radiation Oncology Search Committee
General Surgery Search Committee
Department of Dermatology Search Committee
Department of OB/Gyn Search Committee
Faculty Council, 1996-1999.
Task Force on Interaction with Biomedical Industry, Chairman.
Academic Council

INVITED PRESENTATIONS:

1. Transfer of Plasmacytoma Immunity with Immune RNA Extracts from Tumor-bearing Balb/C mice. American Dental Association, Chicago, IL, 1977.
2. Principles of Cancer Immunology. University of Indiana School of Medicine. Gary, Indiana, 1978.

3. Synergy between Cytotoxic Chemotherapy and Anti-Tumor Immunity in Solid Tumor Cancer Patients. Department of Pathology, University of Illinois Medical Center, Chicago, IL, 1979.
4. Selective Effects of Cytotoxic Chemotherapy on Suppressor Cells in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1980.
5. Immunoregulatory Cell Function and Impaired Immunity in Patients with Gliomas. Department of Neurosurgery, Cook County Hospital, 1983.
6. Effect of Interferon Therapy on Prostaglandin-producing Suppressor Cells in Renal Cell Cancer Patients. Burroughs Wellcome, Research Triangle Park, North Carolina, 1984.
7. Changes in Prostaglandin Metabolism in Monocytes from Interferon-treated Cancer Patients. Department of Microbiology, University of South Florida, Tampa, Florida, 1985.
8. Potentiation of Immunity in Chemotherapy-Treated Cancer Patients. Northwestern School of Medicine, Cancer Center, Chicago, IL, 1985.
9. Prostaglandin-Producing Suppressor Cells in Cancer Patients and AIDS Patients. Department of Pathology, Loyola University School of Medicine, Chicago, IL. 1986.
10. Synergy between Chemotherapy and Immunity in Solid Tumor Cancer Patients. Roswell Park Memorial Institute, Grace Cancer Center, Buffalo, New York, 1986.
11. Modification of the Effects of Cancer Chemotherapy on Immune Responses in Lung Cancer Patients by Treatment with Piroxicam. Pfiser Pharmaceuticals, New Orleans, Louisiana, 1986.
12. Enhancement of Deficient Cellular Immunity in Head and Neck Cancer Patients Treated with Piroxicam. Pfizer Laboratories, Annaheim, California, 1987.
13. Modulation of Immunity in Cancer Patients by Prostaglandin Antagonists. Second International Conference on Immunity to Cancer. Williamsburg, Virginia, 1987.
14. Potential for Combining Cytotoxic Chemotherapy and Biological Response Modifiers in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1988.
15. Monocyte Immunoregulatory Cell Function in HIV-Infected Patients. American Red Cross, Chicago, IL, 1988.

16. Lymphokine Activated Killer Cell Function in Tumor Infiltrating Leukocytes from Colon Cancer Patients. Illinois Cancer Council Symposium on Biological Response Modifiers, Chicago, IL, 1988.
17. Principles of Cancer Immunology. Department of Surgery, Grant Hospital Chicago, IL, 1990.
18. Immune Function in Cancer Patients and the Effects of Chemotherapy. Lederle Laboratories Symposium. Chicago, IL, 1990.
19. Danazol Effects on Peritoneal Macrophage Function in Patients with Endometriosis. Sterling International. New York, NY, 1991.
20. Peritoneal Macrophage Function in Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1991.
21. The Biology and Immunology of Cancer. American College of Surgeons, Chicago, IL, 1991.
22. Immunotherapy of Cancer Patients, Baxter/Bartels Oncology Focus Meeting. Chicago, IL, 1992.
23. Systemic and Local Tumor Immunity in Patients with Solid Tumors. Abbott Laboratories, Abbott Park, IL, 1992.
24. Modulation of Endometrial Cell Proliferation by Monocytes in Patients with Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1992.
25. Modulation of Tumoricidal Function in Tumor-Associated Macrophages from Solid Tumor Patients. Section of Hematology/Oncology, University of Chicago Chicago, IL, 1992.
26. Stimulation of Endometrial Cell Proliferation by Monocytes in Endometriosis Patients. Adeza Biomedical Corp. Sunnyvale, CA. 1993.
27. Cytokine Synthesis by Circulating Monocytes in Patients with Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1994.
28. Effects of Virulizin on Macrophage Functions of Cancer Patients. Hoffman LaRoche, Nutley, NJ, 1994.

29. Macrophage Functional Changes in Unexplained Infertility. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1995.
30. Differential Sensitivity of Ectopic and Eutopic Endometrial Cells to Macrophage-Mediated Cytolysis in Women with Endometriosis. Chicago Association of Reproductive Endocrinology. Chicago, IL, 1996.
31. Effects of Virulizin on Macrophage Functions of Cancer Patients and Women with Endometriosis. Schering Plough, NJ, 1996.
32. Chemotherapy-induced immune modulation in cancer patients. National Cancer Institute of Japan-Tokyo Japan, January, 1997.
33. Macrophage functions in cancer and endometriosis and its modulation by virulizin. Pharmacia. Milano, Italy, February, 1997.
34. Strategies for immune stimulation in cancer patients. Connaught Laboratories, Toronto, Canada, October, 1997.

Exhibit B

1515

Tumor inflammatory response induced by immunization with autologous melanoma cells conjugated to dinitrophenol (DNP). D. Berd, M.J. Mastrangelo, C. Green, C. Clark, and E. Hart. Thomas Jefferson University, Philadelphia, PA 19107.

Treatment of melanoma patients with an autologous vaccine preceded by low dose cyclophosphamide (CY) induces delayed-type hypersensitivity (DTH) to melanoma cells, and in some cases, regression of metastatic tumors. Now, we are attempting to increase the efficiency of the process by immunizing with tumor cells conjugated to the hapten, DNP. Patients with metastatic melanoma were sensitized to DNP by topical application of dinitrochlorobenzene (DNCB). Two weeks later, they were injected with a vaccine consisting of $10 \times 25 \times 10^6$ autologous, irradiated melanoma cells conjugated to DNP and mixed with BCG. CY 300 mg/m² IV was given 3 days before DNCB or vaccine. Of 4 patients evaluable so far, 3 have developed a striking inflammatory response in tumor masses after 2 vaccine treatments (8 weeks). Patient #1 developed erythema and swelling in the >50 large (1-3 cm) dermal metastases on her leg and lower abdomen, followed by ulceration and drainage of necrotic material, and some are beginning to regress. Biopsy showed infiltration with CD4+ and CD8+ T lymphocytes. Patient #2 developed erythema and swelling in the skin of her lower abdomen and groin overlying large (8 cm) nodal masses. These have not yet regressed, but have changed in consistency from rock-hard to fluctuant. Patient #3 exhibited moderate erythema in the skin overlying subcutaneous metastases. All 3 patients have developed DTH to both DNCB and to DNP-conjugated autologous lymphocytes. Although these results are preliminary, they suggest that this new strategy may represent a significant advance in the immunotherapy of human melanoma.

1516

Inhibition of Tumor-Induced Suppressor T Lymphocyte (Ts) Activity by Murine Interferon Beta (IFN- β). Deepak M. Sahasrabudhe, University of Rochester Cancer Center, Rochester, NY, 14642

In some tumor models inhibition of Ts-activity is a prerequisite to successful immunotherapy. Based on our data in the DNFB model (J Exp Med 166:1573, 1987) the effect of IFN- β on P815 mastocytoma-induced Ts-activity was evaluated.

In this model, concomitant antitumor immunity (Tc) peaks by Day 10 and is down regulated by Ts by Day 15. Cytotoxicity generated after a mixed lymphocyte tumor culture (MLTC) correlates with *in vivo* immunity and suppression of cytotoxicity correlates with *in vivo* Ts-activity.

Tumors were initiated by injecting 2×10^6 P815 cells subcutaneously on Day 1. IFN- β (10U, 1000U, 5000U) or buffer were injected i.v. every other day x 6 doses starting on Day 5. On Day 16, MLTC's were set up. Five days later a cytotoxicity assay was performed against 51Cr labelled P815 cells. % specific lysis is shown. Numbers in parenthesis represent the dose of IFN- β .

| E:T | Tc + | | Tc | | Ts | | Tc | | Ts | | Tc | | Ts | |
|------|----------|---------|----|-----|----|-----|----|-----|----|-----|----|-----|----|-----|
| | Tc Naive | Ts + Ts | Ts | +Ts | Ts | +Ts | Ts | +Ts | Ts | +Ts | Ts | +Ts | Ts | +Ts |
| 50:1 | 88 | 81 | 0 | 19 | 6 | 22 | 23 | 20 | 81 | 84 | 81 | 84 | 81 | 84 |
| 25:1 | 84 | 76 | 0 | 12 | 2 | 21 | 1 | 21 | 63 | 75 | 63 | 75 | 63 | 75 |
| 12:1 | 78 | 79 | 2 | 15 | 3 | 24 | 6 | 23 | 58 | 81 | 58 | 81 | 58 | 81 |
| 6:1 | 70 | 69 | 1 | 7 | 0 | 9 | 0 | 20 | 38 | 64 | 38 | 64 | 38 | 64 |
| 3:1 | 56 | 55 | 0 | 8 | 1 | 13 | 0 | 12 | 21 | 48 | 21 | 48 | 21 | 48 |

Treatment with IFN- β 5000U every other day x 6 doses abrogated Ts-activity without adversely affecting cytotoxicity. IFN- β may be a useful adjunct in the immunotherapy of selected tumors.

1517

Anti-idiotype monoclonal antibody immunization therapy of cutaneous T cell lymphoma. Chatterjee, M., Foon, K., Seong, B.K., Barcos, M., and Kohler, H., Roswell Park Mem. Inst., Buffalo, NY 14263, and UCSD, San Diego, CA 92161.

Cutaneous T cell lymphoma (CTCL) is an indolent non-Hodgkin's lymphoma which is not cured by standard therapies once it reaches advanced stage. A novel approach to therapy is to use internal image anti-idiotype (Id) mAb as an antigen (Ag) substitute for the induction of immunity. We have generated anti-Id mAb (Ab2) binding to a hybridoma SN2 (Ab1), which recognizes a unique glycoprotein, gp37, expressed by a subset of human leukemic T cells (J. Immunol. 139:1354, 1987). At least 2 of these Ab2 may indeed carry the internal image of the gp37 Ag (J. Immunol. 141:1398, 1988). Recently, we investigated the distribution of gp37 Ag by a sensitive immunoperoxidase staining method using mAb SN2. SN2 had a high specificity for T-leukemia/lymphoma cells and did not react with any normal adult tissues tested including thymus, lymphocytes, bone marrow cells, spleen, liver, kidney, lung, brain, heart, etc. CTCL cells from 51 out of 6 patients were strongly positive for gp37 Ag with intense surface membrane staining. The binding of radiolabeled SN2 to CTCL cells was studied for inhibition in the presence of the anti-Id mAbs 4EA2 and 4DC6 which mimic the gp37 Ag. Both clones inhibited the binding 100% and 80% respectively at a concentration of 50 ng. We also generated a murine Ab3 mAb (anti-anti-Id) by immunizing mice with the anti-Id mAb (Ab2). This Ab3 mAb reacts with CTCL cells in an identical fashion as the original Ab1 (SN2). Collectively, these data suggest that Ab2 4EA2 and 4DC6 may be useful for active immunotherapy of CTCL patients. We plan to study the CTCL patients in a phase I clinical trial to determine the effects of this type of therapy on various components of the immune system (both humoral and cellular) and try to identify the criteria to select patients who may benefit from anti-idiotype vaccine therapy.

1518

Syngeneic murine monoclonal anti-idiotypes bearing the internal image of a human breast cancer associated antigen. J. Schmitz and H. Ozer. The Dept. of Microbiology, S.U.N.Y. at Buffalo, Buffalo, NY 14214 and the Division of Medical Oncology, The Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

According to Jerne's network theory, some anti-idiotypes (Ab2) mimic external antigens recognized by specific antibodies (Ab1) and may be used in place of antigen for immunization. The murine monoclonal antibody F36/22 (IgG3, κ), specific for ductal carcinoma antigen (DCA) was used to generate syngeneic monoclonal anti-idiotypes bearing the internal image of DCA. Female BALB/c mice were inoculated intraperitoneally every other week with 100 μ g of F36/22 coupled to keyhole limpet hemocyanin; the first time in complete Freund's adjuvant and subsequently in incomplete adjuvant. Splenic lymphocytes were fused with the murine cell line P3X63 Ag8.653 3 days after the fourth immunization using 50% polyethylene glycol (P. W. 3770). Two hybrids, MTO-1 and MTO-2, were selected based on the ability of culture supernatants to bind to F36/22 but not to the control antibody 2A31F6 (IgG3, κ) in an enzyme linked immunosorbent assay (ELISA) and cloned by limiting dilution. Paratope specificity of Ab2 was demonstrated in two ELISA assays. First, the binding of labeled F36/22 to DCA was inhibited 100% and 75% by 1.6 μ g of MTO-2 and MTO-1 respectively. Second, the binding of labeled Ab2 to Ab1 was inhibited by purified DCA. MTO-1 neither enhances nor inhibits the binding of labeled MTO-2 to Ab1 although in the presence of MTO-2, binding of labeled MTO-1 is enhanced by 100% indicating that these Ab2 recognize distinct idiotopes. Rabbits immunized bi-weekly with MTO-1 or MTO-2 developed antibodies that bound specifically to DCA demonstrating that MTO-1 and MTO-2 bear the internal image of DCA. These data suggest that MTO-1 and MTO-2 could potentially be utilized to immunize high risk patients against progression or development of DCA positive tumors.

Exhibit C

TUMOR VACCINATION

Jules E. Harris, MD

Donald P. Braun, PhD

Rush Medical College of Rush University

An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.

Tumor vaccination is an active, specific immunotherapy for malignant disease. It may be defined as "the administration of tumor cells, modified tumor cells, or tumor-cell surface-membrane preparations to stimulate or to augment various components of antitumor immunity to induce tumor regression or to prolong tumor remission achieved by conventional therapy."¹

Vaccines also may be considered a type of biologic response-modifier therapy. The approach is based on the belief that the host is capable of mounting an effective immune response against tumors if appropriately stimulated, a belief that was first advanced around the turn of the century.² The concept of immunologic surveillance, which evolved some 40 to 50 years later, suggested that the human host was capable, under certain circumstances, of rejecting a tumor essentially in the same manner as a homograft was rejected.³

The first attempt to vaccinate humans against cancer was undertaken in 1902.⁴ In

this initial attempt, fluid was extracted from tumors in patients with advanced disease.⁵ Over the next 50 years, a great variety of tumor cell preparations obtained from autologous or allogeneic tumors were used, generally to treat patients with advanced disease.

Fresh interest in the clinical potential of tumor vaccination was stimulated in the 1950s and 1960s by experimental studies conducted in syngeneic rodents. These demonstrated unequivocally that chemically induced and virally induced tumors had both shared and uniquely individual tumor-specific transplantation antigens (TSTAs).

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens (TAAs) rather than against TSTA. Also, TAAs were found on embryonic cells and tumor cells. The "unique antigens" on human tumor cells appear to result from tumor cell dedifferentiation for display of a partial embryonic-cell-membrane antigenic profile. Other TAAs arise as a consequence of the modification of normal "self" antigens producing an "altered self" phenotype.

TABLE 1

Antitumor Immune Mechanisms

- Activated macrophage cytotoxicity
- Cytotoxic T cells ✓
- Natural killer (NK) cells
- Lymphokine-activated killer cells
- Humoral antibodies (complement dependent)
- Antibody-dependent cellular cytotoxicity (complement-independent, macrophage, neutrophil or NK-cell-dependent)

MANY UNRESOLVED CLINICAL ISSUES

The principal human immune responses to tumor antigens are listed in Table 1. It has not been definitively established which of these immune responses, alone or in combination, are the most important in a host response to cancer, nor is it clear which should be targeted for stimulation with tumor vaccination. There are many unresolved issues pertaining to the actual vaccine formulation that need to be addressed; some of these depend on whether the vaccine formulation is based on intact tumor cells (Table 2) or tumor cell extracts or products (Table 3).

In addition to vaccine formulation, several other questions remain:

- Should cellular extracts or whole cells be mixed with immunomodulating adjuvants

to increase tumor vaccine immunogenicity?

■ Should tumor vaccines with or without adjuvants be used alone or in combination with cytotoxic drugs that can modulate or suppress undesirable immune responses?

■ Should cytokines be used to augment immune responses to a vaccine?

■ Is there a place for tumor vaccination in patients with advanced cancer?

■ What is the appropriate dose, schedule, and route of administration for effective tumor vaccination?

■ What measurement or surrogate biologic end point can be used to assess the biologic effectiveness of the vaccine?

■ Will the immune response against TAA produced by a human tumor vaccine be selective and specific for tumor cells or will autoimmune reactions against normal cells be a possible toxicity associated with vaccination?

Advances in molecular genetics and the availability of monoclonal antibody reagents now make it possible to purify cells and cell components with defined and unique antigenic characteristics for use in human tumor vaccines. However, a number of the promising clinical trials of tumor vaccination conducted in the 1970s and 1980s used relatively simple and empiric methods of tumor vaccination preparation.

In one study, surgically resected stage I and II lung cancer patients were treated with a vaccine prepared from allogeneic tumor cells.⁶ Cell membranes from viable tumor cells were subjected to low-frequency sonication and the soluble material separated with Sephadex G-200. Polyacrylamide gel electrophoresis was used to purify protein band material, which could elicit delayed hypersensitivity reactions (DHRs) in lung cancer patients. This material was administered intracutaneously in combination with Freund's complete adjuvant (FCA) in a series of three injections at monthly intervals beginning about 1 month after surgery.

Pilot studies suggested that this form of therapy delayed or prevented tumor recurrence. The approach was tested in a large multicenter clinical trial, which found no difference in survival between control patients and patients treated with FCA alone or with FCA and tumor antigen.⁶ No autoimmune toxicity was noted during the course of these studies. Peripheral blood monocytes producing excessive amounts of prostaglandins appeared in the circulation prior to

TABLE 2

Critical Issues for Whole-Tumor-Cell Vaccines

Should autologous or allogeneic cells be employed?

Should cells be obtained from fresh surgical specimens or from tissue cell lines?

Should cells first be irradiated to maintain their membrane integrity but prevent their proliferation?

How can the reproducibility of vaccine preparation be assured?

How can whole tumor cells be used that are gene modified for the following phenotypic changes (individual or in combination) to enhance immunogenicity: (1) expression of HLA class I or II antigens and/or adhesion molecules; (2) secretion of immunomodulatory stimulating cytokines, such as interleukin-2 or tumor necrosis factor; and (3) secretion of chemotactic cytokines?

clinical relapse in patients who failed in all three arms of the study.

Further analysis of this clinical trial, however, suggests that a survival benefit may have been obtained in the fraction of patients in whom careful attention was paid to thorough homogenization of tumor antigen in the FCA. Vaccinated long-term survivors also may have developed more intense DHRs to tumor antigen. The methods employed in this study, although important and innovative, need to overcome the problematic nature of the technique's purification process and reproducibility (Table 3) before wider application in humans is feasible.

A tumor vaccination study in patients with surgically resected Dukes B₂ through C₂ colorectal cancer was conducted, based on rigorously evaluated preclinical experimental animal data in which requirements for effective immunotherapy were established.⁷ An elegant series of studies of a guinea pig line-10 hepatocarcinoma model showed convincingly that bacillus Calmette-Guérin (BCG) admixed with syngeneic tumor cells could induce sufficient systemic immunity to elimi-

The concept of immunologic surveillance... suggested that the human host was capable under certain circumstances of rejecting a tumor essentially in the same manner as a homograft was rejected.

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens....

nate a limited metastatic disease burden.⁸ These studies controlled for variables such as the number and viability of tumor cells, and ratio of viable BCG organisms to tumor cells. In the pilots that evolved from these trials, patients were randomized to a control arm or were vaccinated with their own tumor cells, obtained from surgical specimens at the time of operation and cryopreserved until thawed and irradiated prior to use.⁹ Treated patients underwent a schedule of three intradermal vaccine treatments weekly beginning 4 to 5 weeks after tumor resection.

The first two vaccine preparations consisted of irradiated cells and BCG; the third vaccine preparation was composed of irradiated tumor cells alone. Vaccinated patients developed augmented DHR to their autologous tumor cells with greater frequency than nonvaccinated patients. A DHR increase to autologous normal intestinal mucosa cells was not seen. An Eastern Cooperative Oncology Group trial is now evaluating this approach for surgically resected Dukes B₂ and B₃ patients.

FIRST TRIALS WITH HUMAN TUMOR VACCINES

These studies, the first truly large, randomized, controlled, multi-institutional clinical

trials of human tumor vaccines for solid tumors, are examples of the use of whole cells and cell extracts for human tumor-vaccine preparation. In each case, an adjuvant substance was added to enhance the immunogenicity of the vaccine.

An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.¹⁰ Patients received cyclophosphamide before the vaccine in an attempt to modulate the activity of suppressor T-lymphocytes. The vaccine was prepared by methods similar to those previously described⁷ and combined with BCG.

Other investigators have also used cyclophosphamide to inhibit suppressor T-lymphocyte activity prior to the administration of a malignant melanoma vaccine.¹¹ The vaccine preparation consisted of mechanically disrupted allogeneic tumor cells from melanoma cell lines. The concentration of vaccine was standardized in the preparation through measurements of a melanoma-associated antigen.

Measurement was performed by binding inhibition enzyme immunoassays using a monoclonal antibody. The vaccine was given subcutaneously with an adjuvant consisting of detoxified endotoxin (monophosphoryl lipid A) mycobacterial cell wall skeleton and squalene oil. In this trial, regressions of disease were seen in patients with metastatic disease.

VIRAL ONCOLYSATES FOR HUMAN TUMOR VACCINATION

The use of viral oncolysates for human tumor vaccination combines the potential immunogenic benefit of whole cells with the value of cell extracts.¹² Viral oncolysates are homogenates of virus-infected cells. The virus in the mixture is believed to have an adjuvant rather than an antigenic role. Allogeneic and autologous viral oncolysates have been used in human immunotherapy. Influenza virus and vaccinia virus have been most frequently used in the preparation of viral oncolysates since the first report of this procedure in 1974.¹³

Pilot studies have suggested a protective or therapeutic benefit for viral oncolysates in gynecologic cancer, melanoma, and sarcoma. However, these reports must be considered anecdotal until larger randomized investigations are conducted.

TABLE 3

Critical Issues for Tumor-Cell-Extract Vaccines

What method of antigen extraction should be employed?

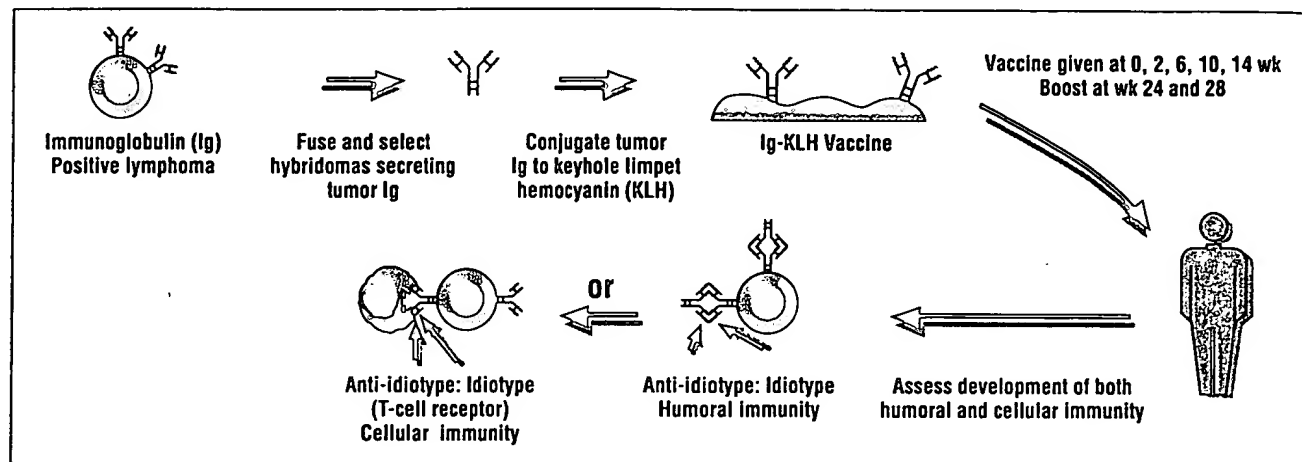
Should material be derived from a single source or should pooled material from a number of sources be used?

What methods are to be used to identify material in cellular extracts that will produce the most effective stimulation of antitumor immune responses?

Should extracted material be separated from HLA antigens that are present on both normal and malignant cells?

How can reproducibility of vaccine preparation be assured?

Mexican



It may be possible to vaccinate against some human cancers by immunizing against those few viruses presently known to be associated with cancer in humans. Hepatitis B virus infection is associated with the development of primary hepatocellular cancer. Immunizing against this virus will prevent its hepatic damage and may reduce the incidence of associated cancer.¹⁴

Finally, one of the most innovative approaches to tumor vaccination that has been developed relies on the use of idiotypic molecules that reiterate the molecular configuration of tumor-associated antigens. This approach is based on principles that predict that the variable regions of immunoglobulins and T-cell receptors that are

responsible for antigen recognition are themselves capable of provoking both B-cell and T-cell immunity.¹⁵

These concepts led other investigators to vaccinate patients who have B-cell lymphoma with the autologous immunoglobulins from each patient's tumor following cytotoxic chemotherapy¹⁶ (Figure). Vaccinated patients developed either humoral immunity, cellular immunity, or both; in the two patients with measurable disease, complete tumor regression was observed. These preliminary results demonstrate the feasibility of idiotypic vaccination for B-cell and T-cell malignant diseases and suggest that similar approaches might also be developed for nonlymphoreticular malignancies.

FIGURE

Strategy for idiotype vaccination. Source: Adapted from Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller R, Levy R. Induction of immune responses in patients with B cell lymphoma against surface immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992; 327:1209-1215.

REFERENCES

- Hersh EM, Marlitz GM, Gutteman GM, and Richman SP. Immunotherapy of human cancer. In: Becker FF, ed. *Cancer: A Comprehensive Treatise*. New York, NY: Plenum Press; 1977; 425.
- Ehrlich P. (1909) The collected papers of Paul Ehrlich. In: Himmelweit F, ed. *Immunology and Cancer Research*. London: Pergamon Press; 1957:550.
- Burnet M. *Cellular Immunology*. Cambridge, England: Cambridge University Press; 1969.
- Von Leydon E, Blumenthal F. Vorläufige mitteilungen über einige ergebnisse der krebsforschung auf der I. Medizinischen Klinik Dtsch. *Med Wochenschr.* 1902;28:637-638.
- Southam CM. Applications of immunology to clinical cancer: past attempts and failure possibilities. *Can Res.* 1961;21:1302-1316.
- Stewart THM, Shelley WE, Willan AR, Hollinshead AC. An evaluation of the role of tumor-specific antigens. In: Mountain C, ed. *Lung Cancer: Current Status and Prospect for the Future*. Houston, Texas: University of Texas Press; 1986: 351.
- Hoover HC, Hanna MG. Immunotherapy by active specific immunization: clinical applications. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Biological Therapy of Cancer*. Philadelphia, Pa: JB Lippincott Company, 1991.
- Hoover HC, Peters LC, Brandhorst JS, et al. Therapy of spontaneous metastases with an autologous tumor vaccine in a guinea pig model. *J Surg Res.* 1981;30:409-415.
- Hoover HC, Surdyke MG; Dangel RB, et al. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. *Cancer.* 1985;55:1236-1243.
- Berd D, Maguire HC Jr, Mastrangelo M. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. *Can Res.* 1986;46:2572-2577.
- Mitchell MS, Harel W, Kempf RA, et al. Active-specific immunotherapy for melanoma. *J Clin Oncol.* 1990;8:856-869.
- Ioannides CG, Platsonas CD, Patenia R, et al. T-Cell functions in ovarian cancer patients treated with viral oncolysates. Increased helper activity to immunoglobulins production. *Anticancer Research.* 1990;10:645-654.
- Sinkovics JG, Williams DE, Campos LT, et al. Intensification of immune reactions of patients to cultured sarcoma cells: attempts at monitored immunotherapy. *Semin Oncol.* 1974;1:351-365.
- Stevenson FK. Tumor vaccine. *FASEB J.* 1991;5:2180-2257.
- Schwartz RS. Therapeutic clonotypic vaccines. *N Engl J Med.* 1992; 327:1236-1237.
- Kwak LW, Campbell MJ, Czerwinski DK, et al. Induction of immune responses in patients with B-cell lymphoma against the surface immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992;327:1209-1215.

Exhibit D

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**

[CANCER RESEARCH 38, 204-208, January 1978]

Immunotherapy of Established Micrometastases with *Bacillus Calmette-Guérin* Tumor Cell Vaccine¹

M. G. Hanna, Jr., and L. C. Peters

Basic Research Program, National Cancer Institute Frederick Cancer Research Center, Frederick, Maryland 21701

ABSTRACT

We evaluated the use of *Bacillus Calmette-Guérin* admixed with tumor cells as a vaccine to induce systemic tumor immunity for therapy of subclinical (micrometastatic) disease. In several experiments inbred strain 2 guinea pigs were given i.v. injections of either 10^4 , 10^5 , or 10^6 syngeneic L10 hepatocarcinoma cells, and initial vaccinations were administered either 1 or 4 days after tumor inoculation. Variables in vaccine preparation, such as ratio of viable *Bacillus Calmette-Guérin* organisms to tumor cells, procedures for freezing the tumor cells, X-ray treatment of tumor cells, and vaccination regimen were evaluated. The studies demonstrated that under defined conditions nontumorigenic vaccines of *Bacillus Calmette-Guérin* and tumor cells can cure the majority of animals of otherwise lethal visceral micrometastases.

INTRODUCTION

The strategy of immunotherapy for cancer in experimental animal models and humans is limited by many factors including the stage, type, and location of the tumor; the level of antigenicity of the tumor cells; and the status of the host immune response. Clinical immunotherapy has been proceeding with relatively limited guidance from experimental animal models. Of the several approaches to immunotherapy of localized tumor and/or disseminated minimal residual tumor, immune potentiation by microbial agents has received the greatest attention. The most encouraging experimental and clinical data to date have resulted from protocols consisting of bacterial vaccines or nonspecific immunostimulants, primarily *Mycobacterium bovis* strain BCG,² administered i.t. (17, 18, 21) or systemically either alone (7, 8, 16) or admixed with tumor cells in the form of a vaccine (22, 23). One impetus for the use of BCG in immunotherapy has been the development of an experimental system that meets some of the requirements of a model to study an established tumor with regional lymph node metastasis (19). It has been demonstrated that regression of transplanted syngeneic hepatocarcinomas growing in the skin of inbred strain 2 guinea pigs and elimination of regional lymph node metastases are achieved in the majority of animals after i.t. injection of viable BCG (12, 26). This particular aspect of immunotherapy in the guinea pig

model, although intriguing, is very limited with respect to the type, stage, and location of the tumor as well as with respect to the route of administration of BCG. Nevertheless, the initial studies established 1 fact that has broad implications. During BCG-mediated tumor regression and elimination of regional lymph node metastases, there is the development of systemic cell-mediated tumor immunity demonstrated by rapid rejection of a second tumor challenge several weeks after BCG treatment (11, 25, 27). This is a very important aspect of the model since it is known that, at the tumor stage when BCG administration is optimally effective, surgical excision of the tumor and regional lymph node would also be curative. However, no significant development of tumor immunity is achieved with surgery alone.

We recently demonstrated the effectiveness of tumor immunity induced by i.t. injections to eliminate artificially produced distant tumor foci (9, 10). This aspect of the BCG therapy model becomes important when one considers that adjuvant immunotherapy has been primarily tested in cancers for which control of primary tumors is available with surgery, radiotherapy, and/or chemotherapy, but where there is a substantial rate of relapse. Recurrence is usually thought to be due to a small number of residual tumor cells. Adjuvant immunotherapy is intended to eradicate the residual tumor cells by enhancing immunological mechanisms. However, based on all that we have learned, the translation to humans of the results of i.t. BCG injection in the guinea pig model would require careful attention to certain aspects of the treatment. These include tumor stage, dose, injection, route, regimen, and source of BCG. This is not always possible in human cancer, for which immunotherapy is often used for advanced cancer after other forms of treatment have failed. In addition, this model is inappropriate for cases in which i.t. injections are not possible.

An important advance in this guinea pig immunotherapy model would be to achieve effective systemic tumor immunity without the i.t. injection of BCG. We have approached this problem by systematically evaluating the ability of vaccines of BCG admixed with tumor cells to eliminate a disseminated tumor burden. Although previous attempts at BCG-tumor cell vaccine immunotherapy both in inbred guinea pigs (3) and in humans (see Ref. 20 for review) have been limited and somewhat discouraging, relatively little has been done to determine the optimal conditions for vaccination. Here we investigate a number of variables such as the ratio of viable BCG organisms to tumor cells, the freezing procedures, the X-ray treatment of cells, and the vaccination regimen. Although these factors cannot possibly be investigated systematically in humans for ethical reasons, they can be studied in the guinea pig model. Our studies demonstrate that, under defined conditions,

¹ Research sponsored by the National Cancer Institute under Contract NO1-CO-25423 with Litton Bionetics, Inc.

² The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; i.t., intratumoral; i.v., intravenous; L10, line 10 hepatocarcinoma cells; HBSS, Hanks' balanced salt solution; i.d., intradermal; PPD, purified protein derivative of *Bacillus Calmette-Guérin*.

Received August 10, 1977; accepted October 17, 1977.

Immunotherapy of Micrometastases

nontumorigenic vaccines of BCG and tumor cells can cure the majority of animals with lethal disseminated tumors established as visceral micrometastases.

MATERIALS AND METHODS

Animals. Inbred male Sewall Wright strain 2 guinea pigs were obtained from the Frederick Cancer Research Center Animal Breeding Section. These guinea pigs were shown to be histocompatible by skin grafting. They were housed 6 to 10 per cage and fed Wayne guinea pig chow and kale; they weighed 400 to 500 g at the beginning of the experiments.

Tumors. Induction of primary hepatocarcinomas in strain 2 guinea pigs after they were fed the water-soluble carcinogen, diethylnitrosamine, was described previously (19). The antigenic and biological properties of the transplantable ascites tumors developed from the primary hepatocarcinomas have also been described (28).

Ascites hepatocarcinoma cells, L10, were harvested and washed 3 times in HBSS and diluted to desired concentrations. One-ml doses of L10, ranging from 10^4 to 10^6 cells/dose, were injected into the dorsal vein of the penis, producing artificial vascular metastasis. Injections of 10^4 cells resulted in the death of approximately 70 to 80% of the animals, whereas 10^5 and 10^6 cells were fatal to all animals. The times of death varied as a function of dose, and all animals died as a result of metastasis to the lung, mediastinal lymph nodes, and hilar lymph nodes with concurrent visceral metastases.

BCG. *M. bovis* strain BCG (Phipps strain TNC 1029) was obtained from the Trudeau Institute (Saranac Lake, N. Y.). Preparations of BCG, stored at -70° , were rapidly thawed in a 37° water bath and diluted to proper concentrations.

Vaccine Preparation. The L10 tumor was maintained by i.p. passage in guinea pigs. Ascites cell preparations were removed and washed in HBSS. The L10 cells used in vaccine preparation were either fresh or frozen and thawed.

In preparation for freezing, the cells were concentrated and suspended in an equal volume of chilled 15% dimethyl sulfoxide plus 10% fetal calf serum-HBSS solution. The final suspension was 2 to 6×10^7 cells/ml. Two-ml aliquots of the L10 cell suspension were frozen at controlled rates in a Linde BF4 Biological Freezer at $-1^\circ/\text{min}$ to the critical freezing point, flash-frozen through the heat of fusion, and continued at $-1^\circ/\text{min}$ to a final temperature of -60° . The rate of freezing was monitored on a Honeywell Electronic III. The vials were stored in liquid nitrogen. The rationale for this method of freezing has been described in detail elsewhere (14, 15). The vials were rapidly thawed in a 37° water bath. Frozen-thawed cells were slowly diluted to 50 ml in HBSS, washed once, and resuspended in preparation for X-irradiation. Suspensions of fresh and frozen-thawed cells were X-irradiated in 50-ml beakers on ice. X-irradiation was performed with a Phillips MG 301 X-irradiation unit at 500 R/min. A total X-irradiation dose of 20,000 R was achieved. Cell viability counts were performed with the use of the trypan blue dye exclusion test, and viability after irradiation of either fresh or frozen-thawed cells was generally 90%, with less than 10% variation between the fresh or frozen-thawed cells.

BCG (10^9 organisms/ml) was added in equal volume to viable L10 (10^8 cells/ml) for a vaccine ratio of 10:1. A vaccination consisted of an i.d. injection of 0.2 ml. For ratios of 1:10, BCG (10^9 organisms/ml) was diluted 1:100 in HBSS, and aliquots were mixed with 10^8 viable L10 cells/ml. These vaccinations also consisted of an i.d. injection of 0.2 ml. All vaccinations were performed less than 1 hr after the BCG-tumor cell mixtures were prepared.

In preliminary vaccination experiments, the L10 cells were irradiated with 12,000 R; however, we noticed that, although this irradiated cell preparation was not tumorigenic when admixed with BCG, it was tumorigenic when administered i.d. in the absence of BCG. We were concerned that any growth of 12,000-R X-irradiated L10 cells in the skin might preempt developing tumor immunity and thus render the treatment ineffective against disseminated tumor. Therefore, 20,000-R X-irradiation was used in all subsequent experiments with L10 cells in BCG-tumor cell vaccines. Animals were given i.v. injections, in the dorsal vein of the penis, of either 10^4 , 10^5 , or 10^6 L10 cells in 1-ml volumes. All vaccinations were given i.d., beginning in the upper right dorsal quadrant. Successive vaccinations were given in different sites or i.l. in the previous vaccination site. Vaccinations were performed either 1 and 7 days or 4 and 10 days after i.v. L10 injection.

RESULTS

An i.v. dose of 10^4 L10 tumor cells does not lead to the death of all guinea pigs. Approximately 25% of the animals will survive clean injections where leakage did not occur to the regional site. This inoculum is the optimal dose for assessing the influence of the nonspecific side effects of vaccination on tumor cell arrest, the extravasation and establishment in organs, and the immunologically specific effects of the vaccine. Thus, at this initial tumor cell dose of 10^4 , vaccinations were performed at either 1 and 7 or 4 and 10 days after i.v. injections of L10.

Several modes of vaccination as well as 2 ratios of viable BCG to tumor cells were tested in guinea pigs given i.v. Injections of 10^4 L10 cells. The BCG-tumor cell ratios were 10^8 BCG or 10^6 BCG admixed with 10^7 L10. These were administered as either a single vaccination, a single injection of BCG-L10 vaccine followed 6 days later by an i.l. injection of L10 into the previous vaccination site, a single injection of BCG-L10 vaccine followed 6 days later by an injection of L10 alone on the opposite side, or 2 separate injections of BCG-L10 vaccine. Also, the efficacy of frozen L10 cells was compared to that of fresh L10 cells. The results are shown in Table 1.

Compared to the untreated tumor-bearing guinea pigs, no significant difference in survival was detected in animals treated with 2 i.d. injections of BCG or tumor cells alone, regardless of whether the initial treatment was performed 1 or 4 days after i.v. injection of L10.

Single BCG + L10 vaccinations at ratios of 1:10 or 10:1 did not confer significantly greater protection than did vaccinations of BCG alone, tumor cells alone, or nontreated controls. Furthermore, these 2 BCG:L10 ratios could not be associated with significant differences in survival of animals given i.v. injections of 10^4 tumor cells, regardless

M. G. Hanna, Jr., and L. C. Peters

Table 1

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells

This experiment was terminated at 280 days after tumor injection; all nontreated controls died by 120 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test

| Treatment ^a | No. of survivors/ total no. of animals/group at following vaccination times after i.v. injection of tumor | |
|---|---|------------------|
| | Days 1 and 7 | Days 4 and 10 |
| None | 3/12 | |
| (10^5 BCG) (10^5 BCG) | 3/12 | 3/10 |
| (10^7 L10) (10^7 L10) | 2/10 | |
| (10^5 BCG + 10^7 L10) ^b | 4/10 | 4/10 |
| (10^5 BCG + 10^7 L10) ^b | 2/10 | |
| (10^5 BCG + 10^7 L10) (10^7 L10 i.i.) | 8/10 | 8/10 |
| (10^5 BCG + 10^7 L10) (10^7 L10 i.i.) | 9/10 | 9/10 |
| (10^5 BCG + 10^7 FL10 ^c) (10^7 FL10 i.i.) | 8/10 | |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 10/10 | 10/10 |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 10/10 | 9/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 10/10 | 10/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 9/10 | 10/10 |

^a Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."

^b Vaccinations were administered as single sequential injections.

^c FL10, frozen-thawed L10.

of the vaccination schedule. Compared to those animals that received single vaccinations of BCG + L10, BCG, or tumor cells alone and compared to the nontreated controls, significant differences in survival were achieved in tumor-bearing guinea pigs that received the second vaccination of either L10 i.i. ($p < 0.03$), L10 on the opposite side ($p < 0.01$), or BCG-L10 mixture ($p < 0.01$). From 80 to 100% of the animals survived in these treatment groups, regardless of whether the initial vaccine was administered 1 or 4 days after i.v. L10 injection. No significant differences in efficacy were detected between fresh L10 cells and frozen L10 cells.

At 280 days, representative groups of the survivors either were tested for tumor immunity by measurement of rejection of i.d. challenge of 10^5 L10 cells or were killed and autopsied for gross and histological examination for residual tumor. None of the animals autopsied had any evidence of residual tumor. Tumor challenge groups varied in their ability to reject contralateral challenge as a function of treatment. All nontreated controls or groups that had been treated with BCG or tumor cells alone failed to reject contralateral challenge, indicating that these animals were not tumor immune at 280 days after treatment. Seventy to 90% of the survivors in the various multiple vaccination groups rejected contralateral challenge; however, no significant difference in tumor immunity, as measured by contralateral challenge, could be detected among these treatment groups. These data demonstrate that animals that survived

after treatment with ineffective modes of vaccination were not tumor immune, whereas significant protection as well as long-term tumor immunity was conferred on those animals that received efficacious modes of vaccination.

Injections of 10^5 or 10^6 syngeneic L10 cells i.v. are routinely fatal in strain 2 guinea pigs. Vaccinations of BCG alone or tumor cell alone conferred no protection in these tumor-bearing guinea pigs when the animals were given vaccinations 1 and 7 days after i.v. tumor inoculation (Table 2). Survival in all treatment groups was a function of the BCG:L10 cell ratio. Without exception, in guinea pigs given 10^5 or 10^6 cells i.v., a vaccine containing BCG:L10 cells in a ratio of 10:1 yielded significant protection, whereas a ratio of 1:10 was ineffective. Thus, the ratio of viable BCG organisms to tumor cells is a critical factor in the efficacy of the vaccine, and a large amount of BCG is beneficial in the initial vaccination. No significant difference in protection could be detected when the group that received a single BCG + L10 vaccination (10:1) was compared to a similar treatment group that received a second i.i. L10 injection. In contrast, survival was achieved in those animals that received a second injection of L10 alone or BCG + L10 on the opposite side ($p < 0.02$ or $p < 0.01$, respectively). In 2 groups of animals given i.v. injections of 10^5 L10, no significant difference in protection was detected when frozen-thawed L10 was used in the vaccine in place of the fresh L10.

One important consideration was whether BCG-immune guinea pigs could generate effective tumor immunity after

Table 2

Survival of guinea pigs given i.v. injections of 10^5 or 10^6 syngeneic L10 hepatocarcinoma cells

These experiments were terminated at 240 days after tumor injection. All nontreated controls in the 10^5 group died by 95 days, and all nontreated controls in the 10^6 group died by 77 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

| Treatment ^a | No. of survivors/ total no. of animals/group at following i.v. tumor cell dose | |
|---|--|--------|
| | 10^5 | 10^6 |
| None | | |
| (10^5 BCG) (10^5 BCG) | 0/10 | 0/10 |
| (10^7 L10) (10^7 L10) | 0/10 | 0/10 |
| (10^5 BCG + 10^7 L10) ^b | 0/10 | 0/10 |
| (10^5 BCG + 10^7 L10) ^b | 1/10 | 0/10 |
| (10^5 BCG + 10^7 L10) ^b | 2/10 | 0/10 |
| (10^5 BCG + 10^7 L10) (10^7 L10 i.i.) | 1/10 | |
| (10^5 BCG + 10^7 L10) (10^7 L10 i.i.) | 5/10 | |
| (10^5 BCG + 10^7 FL10 ^c) (10^7 L10 FL10 i.i.) | 5/10 | |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 1/10 | 0/10 |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 8/10 | 3/10 |
| (10^5 BCG + 10^7 L10) (10^6 BCG + 10^7 L10) | 1/10 | 1/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 9/10 | 8/10 |
| (10^5 BCG + 10^7 FL10) (10^5 BCG + 10^7 FL10) | 9/10 | |

^a Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."

^b Vaccinations were administered as single sequential injections.

^c FL10, frozen-thawed L10.

Immunotherapy of Micrometastases

BCG + L10 vaccination. Normal guinea pigs as well as guinea pigs previously immunized to BCG and shown to be PPD positive by skin testing were given i.v. injections of 10^5 L10 cells. In this particular experiment vaccinations were performed 4 and 10 days after i.v. tumor inoculation. Two modes of vaccination, BCG + L10 (10:1) at Days 4 and 10 and BCG + L10 (10:1) at Day 4 followed by i.l. L10 at Day 10, were compared in PPD-positive and PPD-negative guinea pigs. Regardless of whether or not the animals were PPD positive, the 2 modes of vaccination conferred significant protection ($p < 0.01$) and did not differ significantly (Table 3).

We next investigated whether multiple BCG + L10 vaccinations at either the 10:1 or 1:10 ratios would improve survival in comparison to single or sequential vaccinations. Guinea pigs were given i.v. injections of 10^5 L10 cells and vaccinated on either 1 day, 1 and 7 days, or 1, 7, and 14 days after i.v. tumor inoculation. Treatments consisted of a single vaccination of BCG + L10 followed by L10 alone or by BCG + L10, 2 simultaneous BCG + L10 vaccinations followed by 2 simultaneous injections of L10 alone or of BCG + L10, or 3 sequential vaccinations of BCG + L10. The results are shown in Table 4.

Initial BCG + L10 vaccinations at a ratio of 1:10 were ineffective regardless of the vaccination schedule. Significant protection ($p < 0.01$) was achieved with all initial vaccinations at ratios of 10:1, but no significant increase in survival was achieved by multiple or sequential vaccinations.

DISCUSSION

Vaccines consisting of tumor cells admixed with BCG, under certain defined conditions, are effective in controlling and eliminating micrometastases in a syngeneic guinea pig tumor system, regardless of whether the animals are BCG immune or not. At the outset it should be stated that this experimental model has major limitations in that it is a transplantable tumor established for a short time in normal guinea pigs and in that the system out of necessity does not take into account such factors as individual variations between the biological behavior of tumors of other histolog-

Table 3

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effectiveness of vaccination in BCG-immune guinea pigs

Guinea pigs were given i.d. injections of 10^5 BCG and skin tested with PPD 21 days after immunization; 2 weeks later animals were given i.v. injections of 10^5 L10. The experiment was terminated 270 days after tumor injection, and all nontreated controls died by 128 days after tumor injection. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

| PPD sensitivity | Treatment at 4 and 10 days | Survivors/total no. of animals/group |
|-----------------|---|--------------------------------------|
| - | None | 0 |
| + | None | 0 |
| - | (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 5/10 |
| + | (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 6/10 |
| - | (10^5 BCG + 10^7 L10) (10^7 L10 i.l.) | 2/10 |
| + | (10^5 BCG + 10^7 L10) (10^7 L10 i.l.) | 6/10 |

Table 4

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effect of multiple vaccinations

This experiment was evaluated at 120 days after i.v. tumor injection, and all animals in the nontreated or single vaccination control groups died by 70 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

| Treatment ^a | Survivors/total no. of animals/group |
|---|--------------------------------------|
| None | 0/13 |
| (10^5 BCG + 10^7 L10) ^b | 0/10 |
| (10^5 BCG + 10^7 L10) ^b | 0/10 |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 0/10 |
| 2(10^5 BCG + 10^7 L10) 2(10^7 L10) ^c | 1/9 |
| (10^5 BCG + 10^7 L10) (10^7 L10) | 3/10 |
| 2(10^5 BCG + 10^7 L10) 2(10^7 L10) | 6/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 1/10 |
| 2(10^5 BCG + 10^7 L10) 2(10^5 BCG + 10^7 L10) | 1/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 5/10 |
| 2(10^5 BCG + 10^7 L10) 2(10^5 BCG + 10^7 L10) | 6/10 |
| (10^5 BCG + 10^7 L10) (10^5 BCG + 10^7 L10) | 4/9 |
| (10^5 BCG + 10^7 L10) | |

^a Treatments were administered 6 days apart on opposite sides, as described in "Materials and Methods."

^b Vaccinations were administered as single injections.

^c Two simultaneous injections.

ical types and the limiting factors of the host. Thus, the model may be used to answer only specific questions fundamental to immunotherapy of micrometastasis.

Under natural conditions the development of metastasis is dependent upon an interplay between properties of the host and properties of the tumor cells. The process is highly selective and represents the end point of several destructive events from which few tumor cells survive. Only a few tumor cells within the primary neoplasm may actually invade blood vessels, and of those even fewer will survive in the circulation. Similarly, not all malignant cells that survive transport are successfully arrested, undergo extravasation, etc. Also, tumor cells, in principle, could be susceptible to host immune and nonimmune defense mechanisms that could destroy malignant cells during any of the steps described above (5, 6).

Metastasis was artificially induced in guinea pigs by i.v. injection of L10, and treatment was not started until adequate time had elapsed to ensure extravasation and localization of tumor cells into the parenchyma of visceral organs. No significant difference in the effectiveness of vaccines was found when the treatment was started 1 or 4 days after tumor cell transplantation. It has previously been demonstrated with i.v. injection of B16 melanoma in mice (4) that, between 1 and 4 hr after i.v. transplantation, there is a 50% reduction in the number of arrested tumor cells in the lung, and at 24 hr only 2% of the cells are retained in the lung as a stable metastatic population. Thus, the results of any treatment administered prior to 24 hr after transplantation are impossible to interpret since beneficial effects could be due to prevention of metastasis rather than to treatment. In this study the lack of difference between

M. G. Hanna, Jr., and L. C. Peters

various effective vaccines when treatment was administered 1 or 4 days after transplantation suggests that a therapeutic effect was indeed achieved with these vaccinations.

It was clear from this study that, of the 2 basic BCG-tumor cell vaccines used, the preparation consisting of 10^6 viable BCG admixed with 10^7 tumor cells was more effective over a broad range of increasing initial tumor burdens than was that of 10^6 BCG admixed with 10^7 tumor cells. Although the latter was effective at initial tumor burdens at 10^4 L10, it was ineffective when the initial tumor cell inoculum was increased to 10^5 L10. Whether this 10:1 BCG:tumor cell ratio is critical or simply a function of total BCG cannot be determined from these studies. However, in a study of the effectiveness of BCG-tumor cell mixtures as vaccines against LSTRA murine leukemia (2), it was found that immunity was high (100%) if the BCG:LSTRA ratio was low (either $5 \times 10^4:10^5$ or $5 \times 10^6:10^5$) and that the proportion of immune mice was low (8%) if the BCG:LSTRA ratio was high ($5 \times 10^6:10^5$).

Tumor cells that were frozen by an established procedure used for preservation of bone marrow in transplantation studies and assessed as an optimal procedure in several low-temperature biology studies (for review, see Ref. 24) were equally as effective in the vaccines as fresh tumor cells. This is contrary to the results of Bartlett *et al.* (1) who used glycerol as the freezing additive. Our cells were frozen in dimethyl sulfoxide and fetal calf serum. The striking difference, however, was the percentage of viability after freezing. Cell viability was approximately 90% after freeze-thawing. If for some reason viability fell below 80% during liquid nitrogen storage, the cells were discarded. In our opinion, the trypan blue exclusion test is a very conservative test of cell damage, and any trauma sufficient to render 20% of the cells sensitive to trypan-blue may have severely damaged the remaining cells or altered their antigenicity. The viability of frozen cells in the experiments of Bartlett *et al.* ranged between 40 and 70% as determined by trypan blue exclusion. Thus, the difference in results with frozen L10 cells may be attributed to suboptimal *versus* optimal freezing conditions. Whether the 20,000-R X-irradiation dose of the tumor cells was an important aspect in the preparation of cells in vaccines is not known. However, studies are under way to test this point in this model since it is recognized that the use of 12,000 R is standard procedure for BCG-tumor cell vaccines in humans.

Of the 3 basic vaccination schedules tested, the 2 that were consistently effective for all tumor burdens were 10^6 BCG admixed with 10^7 L10 followed by 10^7 alone on the opposite side or 2 separate injections of 10^6 BCG admixed with 10^7 L10. The fact that BCG was not required in the second injection of the former schedule and the fact that multiple vaccinations did not improve therapy with respect to the latter schedule or with the less effective vaccine (10^6 BCG + 10^7 L10) suggest that the critical aspect of any of the vaccination schedules is the initial dose of BCG. Following the initial treatment with BCG, effective systemic tumor immunity can be achieved with i.d. injections of tumor cells alone at a different site.

A third vaccination schedule, which consisted of reintroduction of the tumor immunogen into the i.d. site previously injected with BCG + tumor cells, was effective at lower

tumor burdens (10^4) but was less effective at initial tumor burdens of 10^5 . This is in contrast to a similar schedule in which the second tumor cell vaccination was in a different i.d. site. The rationale for the second injection of tumor cells in the BCG-infected site was based on the study of Hawrylko (13), in which the dimensions of BCG-potentiated antitumor response against the murine mastocytoma P815 were investigated. One limitation that we found with this procedure was the difficulty in delivering the tumor cell inoculum in the previously infected dermal site. Early ulcerations of these injected dermal sites were limiting with respect to constant delivery of the tumor immunogen in the second injection.

In this study we have shown that visceral micrometastasis induced by i.v. injection of L10 can be cured by the systemic effect of a tumor cell-BCG vaccine. These results confirm our previous studies on the immunological susceptibility of i.v.-injected L10 cells (9, 10). We have now demonstrated that a nontumorigenic vaccine can affect immunotherapy. These results demonstrate that there is a critical dose for BCG in the initial vaccination but that BCG is not essential in the subsequent vaccination and that optimum therapy could be achieved with 2 vaccinations separated by a period of 6 days. Furthermore, the induced tumor immunity, which can cure the majority of guinea pigs with micrometastases is achieved by 2 vaccinations that require a total of 2×10^7 tumor cells (approximately 20 mg of tumor) administered over a period of 1 week. Also, the tumor cells, when frozen under established optimal conditions, maintain immunogenicity and can be used effectively in vaccines.

REFERENCES

1. Bartlett, G. L., Katsilas, D. C., Kreider, J. W., and Purnell, D. M. Immunogenicity of "Viable" Tumor Cells after Storage in Liquid Nitrogen. *Cancer Immunol. Immunotherapy*, 2: 127-133, 1977.
2. Bartlett, G. L., Purnell, D. M., and Kreider, J. W. BCG Inhibition of Murine Leukemia: Local Suppression and Systemic Tumor Immunity Require Different Doses. *Science*, 197: 299-301, 1976.
3. Bartlett, G. L., and Zbar, B. Tumor-specific Vaccine Containing *Mycobacterium bovis* and Tumor Cells: Safety and Efficacy. *J. Natl. Cancer Inst.*, 48: 1709-1726, 1972.
4. Fidler, I. J. Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125 I-5-Iodo-2'-deoxyuridine. *J. Natl. Cancer Inst.*, 45: 773-78, 1970.
5. Fidler, I. J. Patterns of Tumor Cell Arrest and Development. In: L. Weiss (ed.), *Fundamental Aspects of Metastasis*, pp. 276-289, Amsterdam: Elsevier/North-Holland, Inc., 1976.
6. Fidler, I. J., and Nicolson, G. L. Organ Selectivity for Implantation and Growth of B16 Melanoma Variant Tumor Lines. *J. Natl. Cancer Inst.*, 57: 1199-1202, 1976.
7. Gutterman, J. U., Mavligit, G., McBride, C., Frei, E., and Hersh, E. M. Immunoprophylaxis of Malignant Melanoma with Systemic BCG: Study of Strain, Dose and Schedule. *Natl. Cancer Inst. Monograph* 39: 205-212, 1973.
8. Gutterman, J. U., McBride, C., Freireich, E. J., Mavligit, G., Frei, E., and Hersh, E. M. Active Immunotherapy with BCG for Recurrent Malignant Melanoma. *Lancet*, 1: 1208-1212, 1973.
9. Hanna, M. G., Jr., and Peters, L. C. Efficacy of Intraleisional BCG Therapy in Guinea Pigs with Disseminated Tumor. *Cancer*, 36: 1298-1304, 1975.
10. Hanna, M. G., Jr., Peters, L. C., and Fidler, I. J. The Efficacy of BCG-Induced Tumor Immunity in Guinea Pigs with Regional and Systemic Malignancy. *Cancer Immunol. Immunotherapy*, 1: 171-177, 1976.
11. Hanna, M. G., Jr., Snodgrass, M. J., Zbar, B., and Rapp, H. Histopathology of Tumor Regression after Intraleisional Injection of *Mycobacterium bovis*. IV. Development of Immunity to Tumor Cells and BCG. *J. Natl. Cancer Inst.*, 51: 1897-1908, 1973.
12. Hanna, M. G., Jr., Zbar, B., and Rapp, H. J. Histopathology of Tumor

Immunotherapy of Micrometastases

- Regression after Intravesical Injection of *Mycobacterium bovis*. I. Tumor Growth and Metastasis. *J. Natl. Cancer Inst.*, 48: 1441-1455, 1972.
13. Hawryiko, E. Immunopotentiality with BCG: Dimensions of a Specific Antitumor Response. *J. Natl. Cancer Inst.*, 54: 1189-1187, 1975.
 14. Leibo, S. P., Farrant, J., Mazur, P., Hanna, M. G., Jr., and Smith, L. H. Effects of Freezing on Marrow-Stem Cell Suspensions: Interactions of Cooling and Warming Rates in the Presence of PVP, Sucrose and Glycerol. *Cryobiology*, 6: 315-332, 1970.
 15. Mazur, P., Leibo, S. P., Farrant, J., Chu, E. H. Y., Hanna, M. G., Jr., and Smith, L. H. Interactions of Cooling Rate, Warming Rate, and Protective Additive on the Survival of Frozen Mammalian Cells. In: G. E. W. Wolstenholme and M. O'Connor (eds.), *The Frozen Cell*, pp. 69-88. London: Churchill Livingstone, 1970.
 16. McKneally, M. F., Maver, C., and Kausel, H. W. Regional Immunotherapy of Lung Cancer with Intrapleural BCG. *Lancet*, 1: 377-379, 1976.
 17. Morton, D. L., Eilber, E. R., Joseph, W. L., Trahan, E., and Ketcham, A. S. Immunological Factors in Human Sarcomas and Melanomas: A Rational Basis for Immunotherapy. *Ann. Surg.*, 172: 740-748, 1970.
 18. Pinsky, C., Hirshaut, Y., and Oettinger, H. Treatment of Malignant Melanoma by Intratumoral Injection of BCG. *Natl. Cancer Inst. Monograph*, 39: 225-228, 1973.
 19. Rapp, H. J., Churchill, W. H., Jr., Kronman, B. S., Rolley, R. T., Hammond, W. G., and Borsos, T. Antigenicity of a New Diethylnitrosamine Induced Transplantable Guinea Pig Hepatoma: Pathology and Formation of Ascites Variant. *J. Natl. Cancer Inst.*, 41: 1-11, 1968.
 20. Salmon, S. E. Immunotherapy of Cancer: Present Status of Trials in Man. *Cancer Res.*, 37: 1245-1248, 1977.
 21. Seigler, H. F., Shingleton, W. W., Metzgar, R. S., Buckley, C. E., III, and Bergot, T. M. Immunotherapy in Patients with Melanoma. *Ann. Surg.*, 178: 352-359, 1973.
 22. Sokal, J. E., Aungst, C. W., and Grace, J. T., Jr. Immunotherapy of Chronic Myelocytic Leukemia. *Natl. Cancer Inst. Monograph*, 39: 195-198, 1973.
 23. Sokal, J. E., Aungst, C. W., and Han, T. Use of *Bacillus Calmette-Guérin* as Adjuvant in Human Cell Vaccines. *Cancer Res.*, 32: 1584-1589, 1972.
 24. Weiner, R. S., Oldham, R. K., and Schwarzenberg, L. Cryopreservation of Normal and Neoplastic Cells, pp. 1-279. *Proceedings of the International Conference, Institut National de la Santé et de la Recherche Médicale, Ministère de la Santé Publique et de la Sécurité Sociale, Paris, France, 1973.*
 25. Zbar, B., Bernstein, I. D., Bartlett, G. L., Hanna, M. G., Jr., and Rapp, H. J. Immunotherapy of Cancer: Regression of Intradermal Tumors and Prevention of Growth of Lymph Node Metastases after Intravesical Injection of Living *Mycobacterium bovis* (*Bacillus Calmette-Guérin*). *J. Natl. Cancer Inst.*, 49: 119-130, 1972.
 26. Zbar, B., and Tanaka, T. Immunotherapy of Cancer: Regression of Tumors after Intravesical Injection of *Mycobacterium bovis*. *Science*, 172: 271-273, 1971.
 27. Zbar, B., Wépsic, H. T., Borsos, T., and Rapp, H. J. Tumor-Graft Rejection in Syngeneic Guinea Pigs. Evidence for a Two-Step Mechanism. *J. Natl. Cancer Inst.*, 44: 475-481, 1970.
 28. Zbar, B., Wépsic, H. T., Rapp, H. J., Borsos, T., Kronman, B. S., and Churchill, W. H., Jr. Antigenic Specificity of Hepatomas Induced in Strain-2 Guinea Pigs by Diethylnitrosamine. *J. Natl. Cancer Inst.*, 43: 833-841, 1969.

Exhibit E

ESTABLISHMENT OF A TUMOR-SPECIFIC IMMUNOTHERAPY MODEL UTILIZING TNP-REACTIVE HELPER T CELL ACTIVITY AND ITS APPLICATION TO THE AUTOCHTHONOUS TUMOR SYSTEM¹

HIROMI FUJIWARA,² HISAKAZU AOKI, TAKAYUKI YOSHIOKA, SHOHKEN TOMITA,
RYOICHI IkeGAMI, AND TOSHIYUKI HAMAOKA

From the Department of Oncogenesis, Institute for Cancer Research, Osaka University Medical School, 1-1-50 Fukushima,
Fukushima-ku, Osaka, 553, Japan

Preinduction of potent hapten-reactive helper T cell activity and subsequent immunization with hapten-coupled syngeneic tumor cells result in enhanced induction of tumor-specific immunity through T-T cell collaboration between anti-hapten helper T cells and tumor-specific effector T cells. On the basis of this augmenting mechanism, a tumor-specific immunotherapy protocol was established in which a growing tumor regresses by utilizing a potent trinitrophenyl (TNP)-helper T cell activity. C3H/He mice were allowed to generate the amplified (more potent) TNP-helper T cell activity by skin sensitization with trinitrochlorobenzene (TNCB) after treatment with cyclophosphamide. Five weeks later, the mice were inoculated intradermally with syngeneic transplantable X5563 tumor cells. When these cells were injected into X5563 tumor mass, an appreciable number of growing tumors, in the only group of C3H/He mice in which the amplified TNP-helper T cell activity had been generated were observed to regress (regressor mice). These regressor mice were shown to have acquired tumor-specific T cell-mediated immunity. Such immunity was more potent than that acquired in mice whose tumor was simply removed by surgical resection. These results indicate that *in situ* TNP haptenation of the tumor cells in TNP-primed mice can induce the enhanced tumor-specific immunity leading to the regression of a growing tumor. Most importantly, the present study further investigates the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. The results demonstrate that an appreciable percent of growing methylcholanthrene-induced autochthonous tumors regressed by the above TNP immunotherapy protocol. Thus, the present model provides an effective maneuver for tumor-specific immunotherapy in syngeneic transplantation as well as autochthonous tumor systems.

On the basis of the hypothesis of Mitchison (1) concerning manipulations that might augment tumor-specific immunity, numerous attempts to enhance the im-

munogenicity of tumor-associated transplantation antigens (TATA)³ by coupling additional antigenic determinants on the tumor cell surface have been reported (2-6). Helper T cells can collaborate with effector T cell precursors, such as cytotoxic cell precursors, to enhance immune responses against various antigens including TATA (7). If additional determinants coupled onto the tumor cell act as helper determinants, it is therefore conceivable that preinduction of helper T cell activity to these additional determinants could induce much higher anti-TATA immune responses at the time of stimulation of tumor cells conjugated with the corresponding antigenic determinants.

We defined conditions under which enhanced immune resistance to tumors could be generated by preinducing trinitrophenyl (TNP) hapten-reactive T cells, and by subsequently immunizing with TNP-coupled syngeneic tumor cells (8, 9). This system is designed to induce the most efficient generation of tumor-specific effector T cell activity *in vivo* by virtue of the close linkage of hapten-reactive helper T cells and TATA-specific effector precursor T cells in the microenvironment at the time of stimulation with hapten-coupled tumor cells. Our previous results demonstrated that the generation of potent TNP-helper cell activity after elimination of suppressor cell activity was a prerequisite for amplified generation of *in vivo* protective immunity, and a T-T cell interaction mechanism between TNP-helper T cells and anti-TATA effector T cell precursors was thus suggested to be essential to such a phenomenon (10). These results prompted us to establish an immunotherapeutic protocol in tumor-bearing animals in which such potent TNP-helper T cells were used.

In the present study, when TNP was introduced into the tumor mass of tumor-bearing mice in which the amplified TNP-reactive helper T cell activity had been generated, *in situ* trinitrophenylation of tumor cells resulted in a high incidence of complete regression of growing tumors. We demonstrated that the tumor regression was accompanied by the concurrent generation of a potent tumor-specific T cell immunity, suggesting on the above T-T cell collaboration mechanism was functioning in this tumor immunotherapy protocol. More importantly, the present study also investigates whether such an immunotherapeutic potential realized in the TNP-helper

Received for publication January 25, 1984.

Accepted for publication February 23, 1984.

Some of the expenses of publication of this article were defrayed in part by the page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture.

Address correspondence to Dr. Fujiwara.

³ Abbreviations used in this paper: TATA, tumor-associated transplantation antigens; TNCB, trinitrochlorobenzene; MCA, 3-methylcholanthrene; Cy, cyclophosphamide; CTL, cytotoxic T lymphocyte; i.d., intradermal; DTH, delayed-type hypersensitivity.

TABLE I

Comparison of acquisition of tumor-specific immunity after TNP immunotherapy and surgical resection of tumor

| Group | Mice | Incidence of Resistance against Tumor Challenge ^a |
|-------|---|--|
| A | Normal | 0/10 |
| B | After regression of tumor by TNP immunotherapy ^b | 11/12 |
| C | After surgical resection of tumor ^c | 2/10 |

^a Mice were challenged i.d. with 10^5 viable X5563 tumor cells, and incidence of resistance was determined 3 weeks after the tumor challenge.

^b C3H/He mice whose X5563 tumor regressed in the TNP immunotherapy model as shown in Fig. 1 (group E) were used 3 wk after initial tumor implantation.

^c C3H/He mice were inoculated i.d. with 10^5 X5563 tumor cells and growing tumors were surgically resected 7 days later. Mice were used 2 wk after tumor removal.

growth of metastasized tumor cells. Thus, the difference in incidence of anti-X5563 immunity between two groups above indicates more potent anti-X5563 immune resistance was retained in mice whose tumors regressed by virtue of the TNP immunotherapy.

The development of stronger anti-X5563 immune resistance in regressor mice was also confirmed by comparing the tumor-neutralizing activity of spleen cells from these mice to that of mice whose tumors were surgically resected. Winn assay performed with these two groups of spleen cells at a lower spleen to tumor cell ratio

that appreciably stronger tumor-neutralizing activity was generated in the regressor mice by TNP immunotherapy than in the mice that had tumors resected surgically (Table II).

Additional experiments were performed to test the nature and specificity of the effector mechanism acquired by X5563 tumor regressor mice. Winn assays with the use of spleen cells from the regressor mice also demonstrated that these spleen cells resulted in complete neutralization of X5563 tumor cells when admixed, but failed to exhibit 1) tumor neutralization against X5563 tumor after the treatment of the spleen cells with anti-Thy-1.2 plus C (Table III), and 2) tumor neutralization against another syngeneic tumor MH134 hepatoma (Table IV). These results indicate the T cell nature and specificity of anti-X5563 immunity acquired by the regressor mice in the TNP immunotherapy model.

Application of the TNP immunotherapeutic protocol to an autochthonous tumor system. In the process of application of the present tumor-specific immunotherapy model to a chemical carcinogen-induced autochthonous

tumor system, we extended this TNP immunotherapy model to another transplantable, chemical carcinogen-induced tumor (MCH-1-A1) system in which the tumor was recently induced in C3H/He mice by MCA and has been maintained in our laboratory (less than 10 passages *in vivo*). A similar protocol to that performed in the X5563 tumor system was used and the results are illustrated in Figure 2. In this experiment, TNCB injection into the MCH-1-A1 tumor mass from Cy→TNCB-painted mice led to a high incidence of tumor regression, in contrast to the lack of tumor regression when *in situ* TNP modification was performed in mice not primed to TNP. Thus, this TNP immunotherapy system is also applicable to another recently established transplantable, chemical carcinogen-induced fibrosarcoma tumor system.

The successful regression of growing tumors in an MCA-induced transplantable tumor system by using the TNP immunotherapy regimen encouraged us to test the applicability of this immunotherapy protocol to an MCA-induced autochthonous tumor system. The primary tumor was induced in 500 female C3H/HeN mice at 8 wk of age by injecting 0.5 mg MCA in 0.1 ml olive oil subcutaneously. Four weeks after the MCA inoculation, one half of the group of mice received the combined treatment of Cy injection and TNCB painting, which was capable of inducing the amplified TNP-reactive helper T cell activity, and the remainder were untreated. The mice began to develop a primary, subcutaneous tumor about 8 wk after the MCA treatment. At 9 wk after the MCA injection, 20 to 30% of mice in both TNP-helper-positive and -negative groups bore a tumor in the range of 6 to 9 mm in diameter. Histological examination of 10 autochthonous tumors randomly selected (five mice in each group) revealed that all were fibrosarcoma. Mice that did not receive tumor excision were collected and each group was randomly divided into two groups depending on whether mice were treated with the intratumoral injection of 0.15 ml of 1% TNCB. Therefore, the experiment consisted of four groups: group A: MCA injection only; group B: MCA injection→intratumoral TNCB injection; group C: MCA injection→the combined treatment of Cy plus TNCB painting; and group D: MCA injection→the above combined treatment for priming of potent TNP-helper T cells→intratumoral TNCB injection. The tumor growth of four groups of animals is shown in Figure 3. Most tumors in three groups of mice (groups A, B and C), except for only one animal in group B, continued to grow until the animal died, although the growth rate exhibited varied patterns. Importantly, however, an appreciable number (11 of 25)

TABLE II

Comparison of tumor-neutralizing activity between spleen cells from mice after tumor regression after TNP immunotherapy and from mice after surgical resection of tumor

| Spleen Cells from Mice | Spleen:Tumor Cell Ratio | Tumor Growth ^a (mm diam) | | |
|---|-------------------------|-------------------------------------|-----------|------------|
| | | Day 7 | Day 10 | Day 12 |
| Regression of tumor by TNP immunotherapy ^b | 100:1 | 5.8 ± 1.3 | 9.6 ± 1.0 | 13.5 ± 0.3 |
| Surgical resection of tumor ^c | 100:1 | <3.0 | <3.0 | <3.0 |
| | 100:1 | <3.0 | <3.0 | <3.0 |
| | 10:1 | 4.8 ± 0.9 | 9.3 ± 0.9 | 12.5 ± 1.0 |
| Regression of tumor by TNP immunotherapy | 10:1 | <3.0 | <3.0 | 5.0 ± 1.5 |
| After surgical resection of tumor | 10:1 | <3.0 | 7.5 ± 0.5 | 10.3 ± 1.2 |

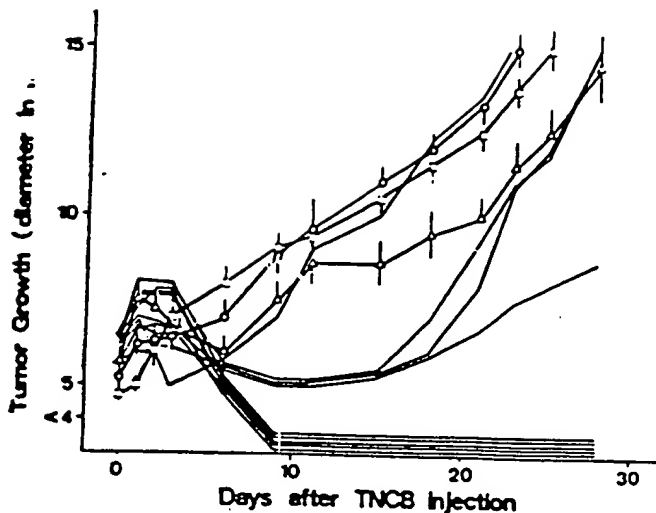


Figure 2. Induction of tumor regression in MCH-1-A1 tumor-bearing mice by using the TNP immunotherapy regimen. C3H/He mice received the combined treatment of Cy injection and TNCB painting. Five weeks after TNCB painting, mice were inoculated i.d. with 10^6 viable MCH-1-A1 tumor cells. The *in situ* TNP haptenation identical to that in Fig. 1 was performed 7 days after tumor cell inoculation. Tumor growth was individually scored and expressed by tumor diameter (—). Tumor growth in control groups was expressed by mean diameter \pm SE of seven mice per group. (O—O), (Δ — Δ), and (□—□) indicate tumor cell inoculation only, tumor cell inoculation—*in situ* TNP haptenation, and the above combined treatment for TNP priming—tumor cell inoculation, respectively.

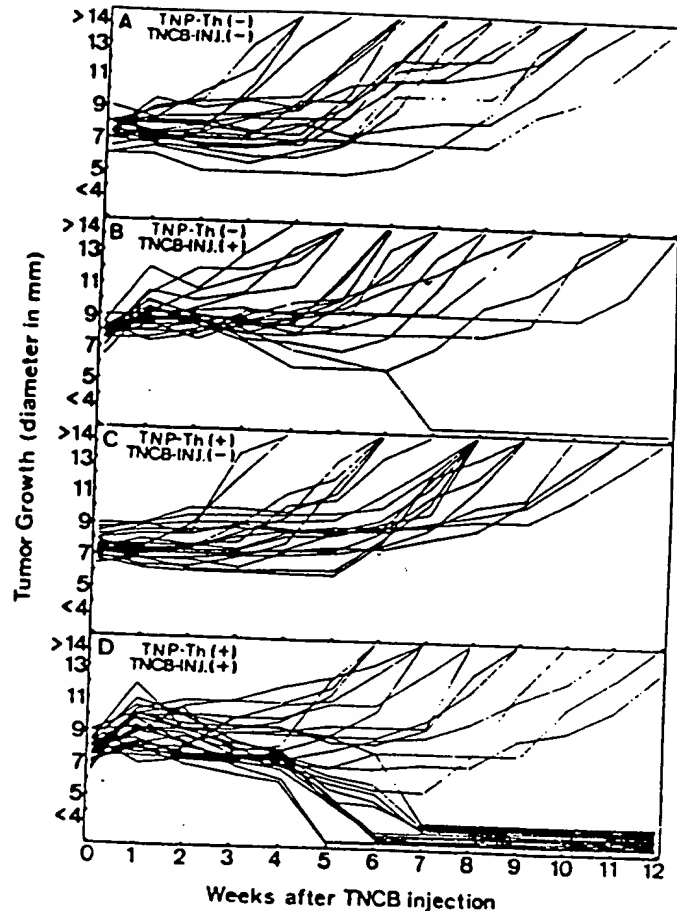


Figure 3. Regression of growing autochthonous tumors by the TNP immunotherapy regimen. C3H/He mice were inoculated subcutaneously with 0.5 mg MCA. Four weeks later, mice received the combined treatment of Cy injection and TNCB painting (groups C and D). Nine weeks after MCA injection, 0.15 ml TNCB in olive oil was administered into subcutaneously growing autochthonous tumors of groups B and D. Group A was MCA inoculation only. Tumor growth was individually scored and expressed by tumor diameter. Tumors in all groups that reached a 14-mm diameter continued to grow for as many as 8 wk ultimately killing the animal. For limitation of the scale, such stage of growth was omitted.

TABLE V
Summary of incidence of tumor regression and mean survival time^a

| Group | Treatment | | Incidence of Tumor Regression | Mean Survival Time (weeks \pm SE) | No. Dead Mice |
|-------|------------------|----------------|-------------------------------|-------------------------------------|---------------|
| | TNP-Th Induction | TNCB Injection | | | |
| A | - | - | 0/20 | 13.00 \pm 0.75 | 20 |
| B | - | + | 1/20 | 11.32 \pm 0.55 | 19 |
| C | + | - | 0/20 | 13.60 \pm 0.58 | 20 |
| D | + | + | 11/25 | 12.90 \pm 0.68 | 14 |

^a Determined 20 wk after injection of TNCB into autochthonous tumor and expressed by mean survival time of dead mice at this stage.

T cell immunity was more potent in the tumor-regressed than in mice whose tumor was surgically resected. It could also be noted that X5563 tumor-specific immunity, which had been acquired in tumor-regressed mice, was mediated by anti-X5563-TATA-specific Lyt-1^+2^- , but not by Lyt-1^+2^+ T cells, indicating that the tumor-specific Lyt-1^+2^- T cell population whose generation was augmented through collaboration with TNP-specific helpers primarily exhibited a protective effect (T. Yoshioka, H. Fujiwara, and T. Hamaoka, manuscript in preparation). Because these Lyt-1^+2^- T cells exhibited no cytotoxic effect on X5563 tumor cells in a 4-hr ^{51}Cr -release assay, further studies are in progress concerning the mechanisms of anti-tumor-specific Lyt-1^+2^- T cell function in eradicating tumor cells *in vivo*.

The most interesting and important finding in the present study [which has not been reported in other tumor-specific immunotherapy experiments] is the demonstration of the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. This finding is worthy of discussion from two perspectives. First, the evidence that TNP immunotherapeutic potential allows the induction of tumor regression to an appreciable proportion in autochthonous as well as transplantable tumor systems clearly emphasizes the validity of this TNP immunotherapy model on the basis of the T-T cell interaction mechanism. This could also provide a theoretical basis for Klein's clinical approaches in which skin malignancies were treated by haptenic reagents (16). Although further investigation is required to explore a chemical suitable for the *in situ* modification of human tumors, the present system may provide a prototype of the immunotherapy of some types of clinical tumors such as skin cancers.

Second, it remains to be proven why 14 of 25 of the autochthonous

tumors bear TATA (17, 18), the qualitative diversity and quantitative heterogeneity in the expression of each putative TATA on an autochthonous tumor cell has not been well determined. Further experiments are therefore required to determine whether the tumor-specific immunity is in fact acquired in mice whose autochthonous tumor has regressed and how putative TATA in each individual autochthonous tumor qualitatively varies, and to investigate the relationship between the immunogenicity of the autochthonous tumor and the prognosis of the tumor-specific immunotherapy. Such approaches are in progress by challenging the autochthonous tumor cells obtained by excisional biopsy

Exhibit F

COMMUNICATION

The Induction of Cytolytic T Lymphocytes with Syngeneic Trinitrophenyl-Coupled Membranes¹

LINDA SHERMAN,² MATTHEW F. MESCHER, AND STEVEN J. BURAKOFF

From the Department of Pathology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Recently we have demonstrated the induction of allogeneic-murine cytolytic T lymphocytes (CTL)³ using purified plasma membranes rather than intact cells as stimulatory agents (1). In this report we extend the use of such subcellular preparations to study the requirements for hapten-specific syngeneic CTL induction. Membranes prepared from trinitrophenyl (TNP) coupled syngeneic tumor cells retain the ability to stimulate both a primary and secondary CTL response. The CTL that are generated are restricted in their lysis to target cells bearing the same H-2 antigens as those present on the TNP-coupled stimulating membranes.

MATERIALS AND METHODS

All materials and methods used in the *in vitro* induction and assay of TNP specific CTL are as previously described (2). Briefly, 7×10^6 spleen cells from nonimmune or immune mice were co-cultured with x-irradiated, TNP-coupled spleen cells or TNP-coupled membranes. After 5 days of culture cells were harvested and cytolytic activity was assessed in a 4-hr assay against 10^4 ⁵¹Cr-labeled TNP-coupled tumor targets or LPS blast cell targets. Immune spleen cells were obtained by priming mice subcutaneously with 2×10^7 TNP-coupled autologous spleen cells 2 weeks before *in vitro* culture. Membranes used in stimulation of CTL were prepared from TNP coupled DBA/2 mastocytoma P815 (H-2^d) tumor cells or from TNP-coupled C57BL/6 (B6) leukemia EL-4 (H-2^b) tumor cells. Purified plasma membranes were used for CTL induction in the experiment described in Table I. The results presented in Tables II and III were obtained by using partially purified plasma membranes referred to as "high speed pellet" in Reference 1. Spontaneous ⁵¹Cr release ranged from 30 to 39% for LPS-induced blast cell targets and from 11 to 19% for tumor cell targets.

Received for publication January 29, 1979.

Accepted for publication March 30, 1979.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by United States Public Health Service Grant CA-14723.

² Present address: Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037.

³ Abbreviations used in this paper: CTL, cytolytic T lymphocytes; H-2, histocompatibility complex-2; B6D2F₁, (C57BL/6 × DBA/2)F₁; B6, C57BL/6.

RESULTS

Plasma membranes prepared from TNP-modified tumor cells were tested for their ability to stimulate both primary and secondary CTL responses by using H-2 syngeneic responder cells. As demonstrated in Tables I and II, such membranes were active in the stimulation of primary and secondary hapten specific CTL. CTL generated by coupled membranes are similar in specificity to those generated by coupled cells in that they preferentially lyse syngeneic target cells (2-4). These membranes stimulated variable amounts of cross-reactive lysis on TNP coupled allogeneic target cells. B6 spleen cells stimulated with TNP-EL-4 membranes did not lyse targets that did not bear TNP. Lytic activity was not induced when B6 spleen cells were co-cultured with uncoupled EL-4 membranes.

It has been recently demonstrated (5, 6) that cells incubated with TNP-coupled proteins are capable of stimulating a hapten-specific cytolytic response that is restricted to target cells that are H-2 identical with the responder cell population. Therefore, it was important to determine if stimulation by the membranes was dependent on the H-2 antigen present on the membrane, or whether the membrane proteins were simply contributing the hapten that was then recognized in conjunction with the H-2 antigens of the responder cell population. B6D2F₁ (H-2^{b/d}) immune spleen cells were stimulated with either TNP-EL-4 membranes or TNP-P815 membranes and the specificity of the resultant CTL was studied. It would be expected that if the H-2 present on the membranes did not influence the specificity of the CTL, then in either case the CTL would lyse both B6-TNP (H-2^b) and B10.D2-TNP (H-2^d) targets to a similar extent. As is shown in Table III the CTL preferentially lyse target cells that bear the same H-2 antigens as the TNP-membranes used in CTL stimulation. Similar specificity was obtained with CTL resulting from stimulation of nonimmune B6D2F₁ spleen cells (Table II). These results indicate that both the TNP and the H-2 antigens present on the membranes determine the specificity of the CTL population.

DISCUSSION

The results described above extend the use of subcellular material to the study of CTL recognition in a chemically modified syngeneic system. The results demonstrate the capacity of membranes prepared from TNP-modified tumor cells to induce primary and secondary CTL having the same specificity as CTL that are induced by TNP-coupled cells. The ability to stimulate B6D2F₁ CTL that are restricted in their recognition to the H-2 antigens present on the stimulating membrane

TABLE I
Specificity of secondary (BALB/c × DBA/2)_F₁ (H-2^d) CTL
stimulated by TNP-coupled membranes

| Stimulator ^a | % Specific ⁵¹ Cr Release | | | |
|-------------------------|-------------------------------------|--------|------------------------------|--------|
| | P815-TNP (H-2 ^b) | | EL-4-TNP (H-2 ^b) | |
| | 25/1 ^c | 12.5/1 | 25/1 | 12.5/1 |
| Experiment 1 | | | | |
| — | 19 | 9 | | |
| 12-μg membranes | 32 | 19 | | |
| 24-μg membranes | 44 | 24 | | |
| 72-μg membranes | 59 | 38 | | |
| Experiment 2 | | | | |
| — | 31 | 19 | 19 | 12 |
| BALB/c-TNP cells | 83 | 61 | 40 | 14 |
| 3-μg membranes | 31 | 16 | 13 | 8 |
| 10-μg membranes | 41 | 19 | 17 | 11 |
| 30-μg membranes | 63 | 40 | 22 | 14 |

^a Membranes used for this experiment were purified plasma membranes obtained from TNP-coupled P815 tumor cells (1).

^c Effector to target ratio.

TABLE II
Induction of primary (C57BL/6 × DBA/2)_F₁ (H-2^{b/d}) CTL by TNP-coupled membranes

| Stimulator ^a | % Specific ⁵¹ Cr Release ^b | |
|-------------------------|--|--------------------------------|
| | B6-TNP (H-2 ^b) | B10.D2-TNP (H-2 ^d) |
| — | 7 | 7 |
| B6-TNP cells | 64 | 34 |
| 24-μg membranes | 28 | 12 |
| 75-μg membranes | 33 | 6 |
| 150-μg membranes | 38 | 6 |

^a Membranes used in this experiment were partially purified from TNP-coupled EL-4 tumor cells (H-2^b).

^b Effector to target ratio is 50:1. Target cells were LPS-stimulated blast cells.

TABLE III
Specificity of TNP-membrane-induced CTL

| Responder | Stimulator | % Specific ⁵¹ Cr Release Targets ^a | |
|---|--------------------------|--|--------------------------------|
| | | B6-TNP (H-2 ^b) | B10.D2-TNP (H-2 ^d) |
| Primed B6D2F ₁ (H-2 ^{b/d}) | — | 6 | 9 |
| | 75 μg EL-4-TNP membranes | 29 | 10 |
| | 84 μg P815-TNP membranes | 16 | 32 |

^a Effector to target ratio was 50:1. Targets were LPS-stimulated blast cells.

preparations indicates that induction results from recognition of both the H-2 and the hapten on the membranes and not from haptenated protein(s) from the membranes that associate with the responder cells. In this regard, TNP membranes are similar in their inductive capacity to TNP cells. It has been previously reported that H-2 antigens need not be directly haptenated in order to obtain a CTL response. Recent experiments that have utilized TNP-coupled serum proteins to stimulate TNP-specific CTL have argued against the contention by Forman *et al.* (7) that only TNP present on H-2 is antigenically active. Although the experiments described above do not address the question of

a requirement for direct haptenization of H-2 to stimulate CTL, it is clear that TNP-membranes are antigenically similar to TNP-cells rather than TNP-proteins.

It is also of interest to consider these results as they address the mechanism of CTL stimulation by subcellular material. The possibility exists that stimulation of CTL by subcellular preparations occurs via presentation of antigen by intact cells present in the cultures (e.g., macrophages) rather than by direct interaction between the membrane vesicle and pre-CTL. It is clear that if indeed material must be presented by viable cells to be antigenic, these cells do not determine the specificity of the resulting CTL.

Ozato and Henney (6) have reported that membranes from TNP-coupled spleen cells failed to induce a secondary syngeneic CTL response whereas the results shown in Tables I and II of this report clearly show that membranes from TNP-coupled tumor cells can induce a specific secondary response. This discrepancy might be accounted for by the difference in cell type used as a membrane source.

The ability to stimulate CTL with TNP-modified membranes opens the possibility that we will be able to isolate, in a soluble form, TNP-modified membrane proteins that retain biologic activity (i.e., the ability to induce CTL) (8). One could then determine whether an effective immunogen is created by TNP-modified non-MHC proteins that interact with H-2, or whether direct chemical modification of H-2 antigens creates the immunogen, or whether both possibilities exist.

SUMMARY

Evidence is presented that trinitrophenyl-coupled tumor membranes are able to induce cytolytic T lymphocytes (CTL) when co-cultured with syngeneic spleen cells. These haptenated membranes stimulate spleen cells from naive and immune mice. The specificity of these CTL is determined by the H-2 antigens of the membranes used for stimulation.

REFERENCES

1. Lemonnier, F., M. Mescher, L. Sherman, and S. Burakoff. 1978. The induction of cytolytic T lymphocytes with purified plasma membranes. *J. Immunol.* 120:1114.
2. Burakoff, S. J., R. N. Germain, and B. Benacerraf. 1976. Cross-reactive lysis of trinitrophenyl (TNP)-derivatized H-2 lymphocytes generated against syngeneic TNP spleen cells. *J. Exp. Med.* 144: 1609.
3. Shearer, G. N., T. G. Rehn, and A. M. Schmitt-Verhulst. 1976. Role of murine major histocompatibility complex in the specificity of *in vitro* T cell-mediated lympholysis against syngeneic trinitrophenyl-modified targets. *Transplant. Rev.* 29:222.
4. Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. *J. Exp. Med.* 142: 403.
5. Schmitt-Verhulst, A. M., C. B. Pettinelli, P. A. Henkart, J. K. Lunney, and G. M. Shearer. 1978. H-2 restricted cytotoxic effectors generated *in vitro* by the addition of trinitrophenyl-conjugated soluble proteins. *J. Exp. Med.* 147:352.
6. Ozato, K., and C. S. Henney. 1978. Studies on lymphocyte-mediated cytotoxicity. XII. Hapten transferred to cell surfaces by interaction with liposomes is recognized by antibody but not by hapten-specific H-2 restricted cytotoxic T cells. *J. Immunol.* 121:2405.
7. Forman, J., E. S. Vitetta, and D. A. Hart. 1977. Relationship between trinitrophenyl and H-2 antigens on trinitrophenyl-modified spleen cells. *J. Immunol.* 118:803.
8. Mescher, M., L. Sherman, F. Lemonnier, and S. Burakoff. 1978. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins. *J. Exp. Med.* 147:946.